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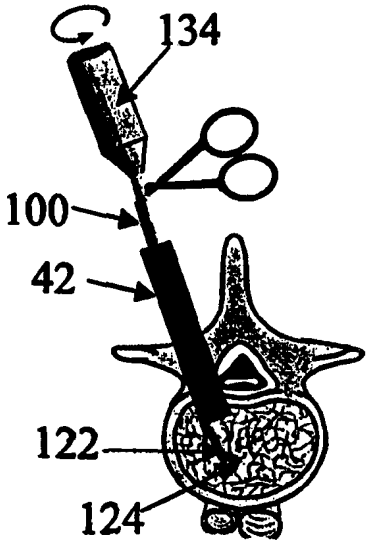
(54) Title: TREATMENT OF BONY DEFECTS WITH OSTEOBLAST PRECURSOR CELLS

(57) Abstract

Healing of bony defects is promoted by suspending osteoblast precursor cells (OPCs) in a porous matrix, which is implanted in the bony defects. The OPCs may be transformed to express a bone morphogenetic protein (BMP), such as BMP–2. Devices are also disclosed for introducing the OPCs into bony defects. One device is a cannula (100) having concentric passageways, such that an endoscope (122) can be introduced through one of the passageways, while the OPCs are introduced through the endoscope or through another passageway without increasing pressure on the OPCs to such an extent that the cells are damaged. A cartridge unit (126) can be inserted through an endoscope to gently advance a cellular suspension through a catheter into the bone.



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TREATMENT OF BONY DEFECTS WITH OSTEOBLAST PRECURSOR CELLS

FIELD OF THE INVENTION

This invention concerns treatments to assist in the healing of bone defects, such as those caused by surgical resection, developmental malformations, trauma or disease. More particularly, the invention concerns therapeutic biological compositions that assist in the repair and regeneration of bone, recombinant DNA techniques for making the compositions, cell lines useful in the method, and devices for delivering and localizing the therapeutic compositions at osseous repair sites.

BACKGROUND OF THE INVENTION

Unlike some other types of fully differentiated tissues, bone has the remarkable ability to regenerate. This regenerative capacity allows broken bones to heal, and has been exploited by surgeons when they perform bone transplants to heal osseous defects left by trauma, congenital malformations and oncologic resections. Bone grafts pose a risk of infection with Crutzfeld-Jacob disease, the human immunodeficiency virus, or other pathogens. There is also an unacceptably high failure rate for autografts (13-30%) and an even greater level of failure for allogeneic preparations (20-35%). These unacceptable clinical outcomes, as well as problems with pathogenic transmissions and immune responses (from allografts), warrant developing safer and more efficient alternatives. These considerations have prompted a search for bioengineered materials that provide a matrix into which bone can grow (osteoconduction), while avoiding the problems of bone grafts.

One proposal has been to use synthetic bone implants as a replacement for human tissue. Acrylic polymers, collagen, silicone elastomers, porous PTFE-carbon fiber composites, calcium phosphate bioceramics (such as biodegradable tricalcium phosphate and hydroxyapatite), and resorbable lactide polymers have all been suggested as possible materials that will provide a porous matrix into which bone can grow. However none of these materials appears to induce significant bone formation (osteogenesis) and are therefore said to lack osteoinduction.

Another problem with bone transplants and synthetic implants is that they are often poorly effective in the elderly, which is the population in which most bone trauma occurs. As the body ages, it loses some of its capacity to regenerate bone, which is essential if the transplant or implant is to be incorporated into the healing osseous defect. A related clinical manifestation of this impaired ability to regulate bone formation is osteoporosis, which often results in debilitating bone and spinal injuries. This disease is thought to be caused by an imbalance between bone formation (by osteoblasts) and bone resorption (by osteoclasts), perhaps arising from a failure of complex cell to cell interactions that maintain this system in homeostasis.

The molecular basis of the process of bone formation has been the subject of intense research during the last few decades. A group of regulator molecules known as bone morphogenetic proteins (BMPs) has been found to direct the cellular processes that form bone during embryogenesis, maintenance and repair. The BMPs are categorized within the transforming growth factor beta (TGF-β) superfamily. The BMPs within this family include BMPs 2-15 (BMP-1 is not part of the TGF-β superfamily). The sequences of BMPs 2-9 were described by Wozney et al. in *Prog. Growth Factors* 1:267-280 (1989), *Mol. Reprod. Dev.* 32:160-167 (1992); and *Science* 242:1528-1534 (1988); and Wang et al. in *Proc. Natl. Acad. Sci.* USA 85:9484-9488 (1988).

The identification and cloning of these BMPs was performed by isolating 16-18 kD polypeptides from bovine bone, digesting them with trypsin, determining the amino acid sequences, and synthesizing oligonucleotide probes. The probes were then used to screen bovine genomic sequence libraries or cDNA libraries, and recombinant clones were identified and used to screen human cDNA libraries to derive recombinant clones that encoded human BMPs; Wang, *Proc. Natl. Acad. Sci USA* 85:9484 (1988); Wozney et al., *Science* 242:1528 (1988). Using this strategy, BMP-1 through BMP-9 were obtained, and their amino acid sequences deduced. Amino acid sequence conservation has allowed BMP-2 through BMP-9 to be partitioned into several subfamilies: BMP-2 and BMP-4; BMP-3 (referred to as osteogenin); BMP-5 through BMP 8 (where BMP-7 and BMP-8 are respectively referred to as osteogenic protein 1 (OP-1) and osteogenic protein 2 (OP-2)); and BMP-9.

More recently, the BMPs 10-15 have been identified with hybridization and polymerase chain reaction technology by Inada et al., *Biochem. Biophys. Res. Commun.* 222:317-322 (1996); Celeste et al., *J. Bone Miner. Res.* 10(1S) 334-339 (1995); and Dube et al., *J. Bone Miner. Res.* 10:333-339 (1995). BMP-12 and BMP-13 appear to be the human homologues of mouse growth/differentiation factor (GDF-7 and GDF-6, respectively).

The numerous patents that have issued on BMPs are a further testament to the intensity of the research in this field. U.S. Patent No. 4,455,256 (Urist) disclosed a general method of making BMP by demineralizing bone tissue, extracting BMP in a solubilizing agent, and precipitating the BMP. The sequences of purified BMP-1 proteins, and DNA sequences encoding them, were disclosed in U.S. Patent No. 5,108,922 (Wang et al.). U.S. Patent Nos. 5,166,058 (Wang et al.) and 5,318,898 (Israel) concerned a process for producing recombinant BMP-2, U.S. Patent No. 5,618,924 (Wang et al.) disclosed BMP-2 products, while U.S. Patent No. 5,670,338 (Murakami et al.) disclosed a process for cloning DNA coding for BMP-2A. DNA sequences encoding proteins for BMP-3 were disclosed in U.S. Patent No. 5,116,738 (Wang et al.), BMP-5 in U.S. Patent No. 5,106,748 (Wozney et al.), BMP-6 in U.S. Patent No. 5,187,076 (Wozney et al.), and BMP-7 in U.S. Patent No. 5,141,905 (Rosen et al.), BMP-8 in PCT Publication WO 91/18098, and BMP-9 in PCT Publication WO 93/00432. Screening techniques for identifying and

evaluating compounds that stimulate bone growth is discussed in WO 96/38590 (Harris et al.). Sequences encoding a broad variety of the BMPs are therefore known, and the term "bone morphogenetic protein" encompasses a variety of peptides.

Properties, roles and anatomic locations of many of the BMPs are summarized in TABLE 1.

TABLE 1

BMP	Properties, Roles and Locations
BMP-1	protease (member of astacin family); may function as a procollagen C-proteinase responsible for removing carboxyl propeptides from procollagens I, II and III; activates BMPs; not osteoinductive; may be involved with Langer-Giedon syndrome; *Drosophila* colloid gene homologue; dorso-ventral fetal patterning
BMP-2	osteoinduction and embryogenesis; fetal formation; differentiation of osteoblasts, adipocytes, and chondrocytes; may influence osteoclast activity and neuronal differentiation; located in bone, spleen, liver, brain, kidney, heart, placenta, and regulates repair in long bone, alveolar clefts, spine fusions, and maxillary sinus augmentation, among others.
BMP-3	osteoinductive; promotes chondrogenic phenotype; located in lung, kidney, brain, intestine. Also known as osteogenin.
BMP-4	osteoinductive; found in apical ectodermal ridge, meninges, lung, kidney, liver; during embryogenesis it is involved in gastrulation and mesoderm formation; produced by dorsal aorta; involved in fracture repair; over-expression associated with ectopic ossification of fibrodysplasia ossificans progressiva.
BMP-5	osteoinductive; found in lung, kidney, liver; embryogenesis
BMP-6	not osteoinductive; involved in embryogenesis, neuronal maturation, and chondrocyte differentiation; found in lung, brain, kidney, uterus, muscle, skin.
BMP-7	osteoinductive; found in adrenal glands, bladder, brain, eye, heart, kidney, lung, placenta, spleen, skeletal muscle; involved in embryogenesis, and repair of long bone, alveolar bone, and spine fusion; induces differentiation of osteoblasts, chondroblasts, adipocytes. Also known as osteogenic protein-1.
BMP-8	initiation and maintenance of spermatogenesis (mouse). Also known as osteogenic protein-2.
BMP-8B	initiation and maintenance of spermatogenesis (mouse); also known as osteogenic protein-3.
BMP-9	osteoinductive; stimulates hepatocyte proliferation; hepatocyte growth and function.
BMP-12 and BMP-13	inhibits terminal differentiation of myoblasts

The discovery of the BMPs was greeted with great expectations about the use of exogenous BMP to help regenerate bone in osseous defects. However, it has been more difficult than initially anticipated to clinically harness their osteogenic activity. Part of this disappointment has been the difficulty of finding a delivery system that provides the appropriate biological milieu for the BMPs to exert their osteogenic effects. In the absence of an appropriate carrier, BMP rapidly diffuses away from its anatomic site of intended use, and its concentration is too low to exert its desired biological activity on mesenchymal cells. Kim et al., *J. Biomed. Material. Res.* 35:279-285 (1997), suggested overcoming this problem by *ex vivo* stimulation of osteoblastic cells with recombinant human BMP-2 (rhBMP-2), followed by grafting of the stimulated cells into areas of bony non-union. However the authors reported no apparent stimulation of osteoblasts using this technique. Ripamonti et al., *South African J. Science* 91:277-280 (1995) noted that development of an effective carrier for BMPs had been hampered by the inability to find a complementary, biocompatible, nonimmunogenic, carvable substrate that provides mechanical support while promoting rapid mesenchymal and vascular invasion.

Proposals have been made to provide osteogenic proteins in a polymer matrix that can be implanted into a bony defect. Polymer matrices made of acrylic esters (U.S. Patent No. 4,526,909, Urist) or lactic acid polymer (U.S. Patent No. 4,563,489, Urist) have been proposed as carriers for osteogenic proteins. In U.S. Patent No. 4,968,590, Kuber Sampath et al. disclosed the use of matrix polymers made of copolymers and homopolymers of glycolic acid and lactic acid, as well as hydroxyapatite, and calcium phosphates. U.S. Patent No. 5,266,683 (Opperman et al.) discloses a matrix made up of particles of porous materials, with a particle size of 70-850 μm, where the matrix material was collagen, polymers of glycolic acid, lactic acid and butyric acid, and ceramics, such as hydroxyapatite, tricalcium phosphate, and others. The use of poly(α-hydroxy acids) as carriers for bone morphogenetic proteins was disclosed in Hollinger and Leong, *Biomaterials* 17:187-194 (1996). Calcium phosphate or calcium acetate matrices were disclosed in U.S. Patent Nos. 4,789,732 (Urist); 5,306,303 (Lynch); and 5,385,887 (Yim et al.). A collagen containing matrix was suggested in U.S. Patent Nos. 4,975,527 (Koezuka et al.) and 5,531,791 (Wolfinbarger).

Delivery systems for BMPs were reviewed in Mayer et al., *Plastic and Reconstruc. Surg.* 98:247-259 (1996), where it was noted that a collagen delivery system may not be ideal because of the immunologic potential of that material. Similarly, a system that included BMP-2 and autologous blood to poly(lactide-co-glycolide) particles was found to be dislodged by soft tissue movement and oozing from the recipient bone bed, which prevented localization of an effective dose of BMP-2 at the recipient site. Hence, even after years of research, there is still a need for a delivery system that is biocompatible, non-immunogenic, and which provides a sufficient matrix to suspend and localize osteogenic factors, while simultaneously permitting the ingrowth of blood

vessels and providing a structure into which bone can grow, without impeding the final formation of new bone in the defect. When BMP-2 has been used clinically to regenerate bone, massive doses of it have been required to overcome some of these problems, and the result still has not been clinically useful.

Intense scientific attention has also focused on the osteoblast precursor cells (OPCs), and their role in bone formation. Methods for isolating OPCs from homogeneous preparations of stromal cells were described by Rickard et al., *J. Bone Min. Res.* 11:312-324 (1996). Immortalization of OPCs has been disclosed by Evans et al., *J. Ortho. Res.* 13:317-324 (1995); Harris et al., *J. Bone Min. Res.* 10:178-186 (1995); and U.S. Patent No. 5,693,511 (Harris et al.). The use of osteoprogenitor cells has also been discussed as targets for *ex vivo* gene transfer in Onyia et al., *J. Bone Min. Res.* 13:20-30 (1998), while the stimulation of bone by direct transfer of osteogenic plasmid genes into fibroblasts was proposed by Fang et al., *Proc. Natl. Acad. Sci.* USA 93:5753-5758 (1996). Expression of BMP-2 and BMP-4 in mesenchymal C3H10T½ cells was discussed in Ahrens et al., *DNA and Cell Bio.* 10:871-880 (1993), while expression of BMP-2 in Chinese Hamster Ovary Cells was disclosed in Israel et al., *Growth Factors* 7:139-150 (1992).

In spite of many incremental advancements in the understanding of the biology of bone repair, a clinically useful technique for applying these concepts has been elusive.

It is therefore an object of this invention to provide an effective method for the repair of bony defects which can be clinically applied to repair osseous defects.

SUMMARY OF THE INVENTION

The foregoing object may be achieved by providing a therapeutically effective amount of OPCs substantially immobilized in or adjacent to a porous matrix, which is implanted into a bony defect to repair the defect. Alternatively, the invention includes a method in which a bone morphogenetic protein (BMP) is expressed in an OPC, for example the expression of BMP-2 in a conditionally immortalized OPC that is implanted into a bony defect. The OPC which produces the BMP may be immobilized in or adjacent to a porous matrix that maintains the OPC at the site of implantation, and the OPC is responsive to the BMP it expresses so that it produces bone at the site of the osseous defect. The exogenous supply of OPCs also boosts the bone making capability of an ill or aged individual in whom OPCs may be numerically deficient or functionally impaired. The invention also includes methods of transfecting OPCs to express BMP, and administering a therapeutically effective amount of those OPCs to express BMP in effective amounts to repair a bony defect.

The OPCs (either with or without transfection) may be administered in an implant which provides an environment that cushions the OPCs during implantation, and provides a degradable support matrix that localizes the OPCs to heal the bony defect. The implant is also suitable to allow or promote vascular ingrowth and bone formation, without becoming a physical

barrier to the progression of bone formation. In particular embodiments, the implant degrades at a rate that is proportionate to bone formation at the site of localization, so that the implant will degrade as the bone is formed. The degrading implant may also be designed to release a substance that is toxic to the OPCs once bone formation has been substantially promoted or completed.

The implant may include a cell suspension component (for example a gel suspension such as a hydrogel) in which the OPCs are suspended for protection and growth. The implant also includes a porous support component, such as a degradable, substantially biocompatible and non-immunogenic material, for example a poly(α-hydroxy acid) (PHA), such as homopolymers of polylactide (PL), polyglycolide (PG), and their copolymers of poly(lactide-co-glycolide) (PLG). The support component provides a relatively rigid environment that supports soft tissue, protects the gel component, and provides a biological template into which bone growth may occur. The support component is disposed in protective relationship to the suspension component, for example as a contiguous cortex surrounding a gel core, or as an adjacent layer in a laminate or multi-laminate implant. The support component may also include a therapeutically effective amount of a BMP to activate the OPCs in the suspension component. When suspended in the BMP-impregnated support component, the OPCs in particular embodiments have been transformed to express physiologic or supraphysiologic doses of BMP. However, the invention also includes native OPC cells that have not been engineered to express the BMP, but which may be exposed to BMP impregnated in the matrix of the porous support component, as well as OPCs that have not been engineered to express BMP, and are not in a BMP impregnated matrix.

The invention also includes methods of administering OPCs, such as cells expressing supraphysiologic amounts of an osteogenic BMP, such as OPCs into which have been introduced an expression vector for the production of a BMP, such as BMP-2. The OPCs are introduced into a bony defect, such as a traumatic or congenital defect, or an area of deficient bone formation or density (osteopenic bone), as occurs for example in the spine of a person with osteoporosis. The OPCs may be administered in combination with the implant, which provides both protection for the OPCs and a structural matrix in which bone formation occurs. When used to treat osteoporosis, either a cellular suspension or the implant is introduced into a recipient bed of osteoporotic or osteopenic bone to promote new bone formation. The recipient bed may be prepared, for example, by introducing a catheter into the osteoporotic bone and producing a local void or cavity in the bone, into which the cells or implant can be introduced without creating excessive back pressure.

The invention also includes a porous matrix that includes a therapeutically effective amount of a cell that is committed to an osteogenic lineage, such as an OPC. The cell that is committed to an osteogenic lineage can include a conditionally immortalized osteoblast precursor cell having the characteristics of cell line OPC1. In specific embodiments, the porous matrix may be made of a combination of poly(D,L-lactide) and collagen. In other embodiments, the

compositions also contain a therapeutically effective amount of a BMP, more specifically BMP-2. Further embodiments include recombinant BMP-2 expressed by OPC1.

A delivery device for introducing the cellular suspension or implant into the body includes a catheter through which the suspension or implant can be propelled to its desired location. The implant may be designed to conform to the walls of the catheter, for example by making the implant cylindrical when used with a tubular catheter. The cylindrical implant can be formed by providing a cylindrical support cortex around a gel OPC suspension core, or by bending a laminate implant into a cylindrical form for introduction into a tubular catheter. Implants of other shapes can also be used, and made to conform to the shape of a bony defect into which the implant is placed. The implant can also be surgically implanted, without the delivery device.

The delivery device can be a fiber-optic endoscope, which may be used during minimally invasive surgery to locate and treat a bony defect, such as an osteopenic spine. The endoscope may have a distal end with a cavity-forming tip that can be enlarged after introduction of the tip into the bone (for example by inflation of a balloon catheter tip), to perform an "osteoplasty" by compressing surrounding osteopenic bone, and creating the cavity into which the OPCs are to be placed. After the cavity is formed, the balloon catheter is deflated, and the implant is introduced under pressure through the catheter to be deposited in the enlarged cavity. The dimensions of the cavity may be substantially the same (or slightly larger) than the implant, so that the implant substantially fills the cavity. Alternatively, the OPCs can be gently introduced into the cavity, without disrupting the cells in the suspension. The gentle introduction can be accomplished using an auger that extends through the endoscope. Introducing the OPCs into the area of osteopenic bone not only helps form new bone to fill in the cavity, but the BMP expressing OPCs also recruit native OPCs in the patient's body to the site of the osteopenic bone, to encourage additional bone formation in the surrounding bone.

The invention also includes novel cell lines (such as OPC1) that are BMP responsive and which also express exogenous or supraphysiologic concentrations of BMP, recombinant methods for producing such cell lines and rendering them conditionally immortal, compositions incorporating the OPCs, and methods of making the implants. Particular disclosed OPCs can begin bone formation without addition of ascorbic acid and glycerophosphate to culture medium, and may exhibit contact inhibition (so that there is an absence of cellular proliferation after confluence that indicates an absence of oncologic potential).

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description of a preferred embodiment which proceeds with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic view of a method of making a cortex core device (CCD) implant in accordance with the invention, and implanting it into a critical-sized defect in a mandible.

FIG. 2 is a schematic view of a method of making an integrated polymer laminate (IPL) embodiment of the implant, and implanting it into a critical-sized defect in a mandible.

FIG. 3 is a schematic diagram of the plasmid pMX1-sv40T-Neo-195.

FIGS. 4A, 4B, 4C, and 4D are schematic views of several steps in a disclosed method for introducing OPCs into an osteoporotic spine.

FIG. 5 is a top view of a vertebral body, illustrating a hollow cannula introduced through the vertebral pedicle, with an endoscope and a balloon catheter introduced through the cannula.

FIG. 6 is a view, similar to FIG. 5, but showing the balloon catheter inflated to compress surrounding osteopenic vertebral bone.

FIG. 7 is a schematic diagram of the plasmid hCNTF-pNUT-DNT.

FIG. 8 is a histogram representing the radiomorphometric analysis of bone healing in calvarial critical-sized defects in athymic rats, receiving one of four treatments: PLC alone, PLC with OPCs, PLC with rhBMP-2, or PLC, OPCs and rhBMP-2.

FIG. 9 is a histogram representing the histomorphometric analysis of bone healing in calvarial critical-sized defects in athymic rats, receiving one of four treatments: PLC alone, PLC with OPCs, PLC with rhBMP-2, or PLC, OPCs and rhBMP-2.

FIG. 10A is a schematic diagram of a cannula in which an inner channel provides access for an endoscope, and a coaxial outer channel provides a structure through which the OPCs can be introduced.

FIG. 10B is a schematic diagram that shows a cartridge unit for delivery of OPCs using a auger mechanism.

FIG. 10C is a schematic diagram that shows an endoscope inserted through the cannula of FIG. 10A.

FIG. 10D is a schematic diagram that shows the cartridge unit of FIG. 10B inserted into the cannula of FIG. 10A, for controlled delivery of OPCs from the cartridge through the auger mechanism into the bone.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

SEQ. ID. NO. 1: PCR primer to phenotype OPC cells for osteocalcin expression.

SEQ. ID. NO. 2: PCR primer to phenotype OPC cells for osteocalcin expression.
SEQ. ID. NO. 3: PCR primer to phenotype OPC cells for osteonectin expression.
SEQ. ID. NO. 4: PCR primer to phenotype OPC cells for osteonectin expression.
SEQ. ID. NO. 5: PCR primer to phenotype OPC cells for osteopontin expression.
SEQ. ID. NO. 6: PCR primer to phenotype OPC cells for osteopontin expression.
SEQ. ID. NO. 7: PCR primer to phenotype OPC cells for PTH-Receptor expression.
SEQ. ID. NO. 8: PCR primer to phenotype OPC cells for PTH-Receptor expression.
SEQ. ID. NO. 9: PCR primer to phenotype OPC cells for alkaline phosphatase expression.
SEQ. ID. NO. 10: PCR primer to phenotype OPC cells for alkaline phosphatase expression.
SEQ. ID. NO. 11: PCR primer to phenotype OPC cells for procollagen I expression.
SEQ. ID. NO. 12: PCR primer to phenotype OPC cells for procollagen I expression.
SEQ. ID. NO. 13. Nucleotide sequence of KS-hBMP-2 plasmid vector.
SEQ. ID. NO. 14. Nucleotide sequence of IgSP-NS-hBMP-2 plasmid vector.
SEQ. ID. NO. 15. Nucleotide sequence of IgSP-KR-hBMP-2 plasmid vector.
SEQ. ID. NO. 16. Nucleotide sequence of IgSP-RRRR -hBMP-2 plasmid vector.

DETAILED DESCRIPTION

TABLE 2

Abbreviations and Definitions

BMP	Bone morphogenetic protein
CCD	Cortex core device
CSD	Critical-sized defect (a bone defect sufficiently large that it does not spontaneously heal). A CSD in the long bone is considered 2-3X's the diaphyseal diameter.
IPL	Integrated polymer laminate
OPC	Osteoblast precursor cells
Osteoconduction	Ingrowth of vascular tissue from host margins followed by new bone formation (osteoinduction)
PHA	Poly(α-hydroxy acids)
PG	Polyglycolide
PL	Poylactide
PLG	Poly (lactide-co-glycolide)

Isolated: An "isolated" biological component (such as a nucleic acid molecule, protein or organelle) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, *i.e.*, other chromosomal and extra-chromosomal DNA and RNA, proteins and organelles. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Oligonucleotide: A linear polynucleotide sequence of between six and 300 nucleotide

bases in length.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

ORF (open reading frame): A series of nucleotide triplets (codons) coding for amino acids without any internal termination codons. These sequences are usually translatable into a peptide.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers useful in this invention are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the fusion proteins herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (*e.g.*, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified fusion protein preparation is one in which the fusion protein is more enriched than the protein is in its generative environment, for instance within a cell or in a biochemical reaction chamber. Preferably, a preparation of fusion protein is purified such that the fusion protein represents at least 50% of the total protein content of the preparation.

Recombinant: A recombinant nucleic acid molecule is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination can be accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, *e.g.*, by genetic engineering techniques.

Similarly, a recombinant protein is one encoded for by a recombinant nucleic acid molecule.

Sequence identity: The similarity between two nucleic acid sequences, or two amino acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as

sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs of the bispecific fusion protein will possess a relatively high degree of sequence identity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman (*Adv. Appl. Math.* **2**: 482, 1981); Needleman and Wunsch (*J. Mol. Biol.* **48**: 443, 1970); Pearson and Lipman (*PNAS.* USA **85**: 2444, 1988); Higgins and Sharp (*Gene*, **73**: 237-244, 1988); Higgins and Sharp (*CABIOS* **5**: 151-153, 1989); Corpet *et al.* (*Nuc. Acids Res.* **16**: 10881-90, 1988); Huang *et al.* (*Comp. Appls Biosci.* **8**: 155-65, 1992); and Pearson *et al.* (*Methods in Molecular Biology* **24**: 307-31, 1994). Altschul *et al.* (*Nature Genet.*, **6**: 119-29, 1994) presents a detailed consideration of sequence alignment methods and homology calculations.

The alignment tools ALIGN (Myers and Miller, *CABIOS* **4**:11-17, 1989) or LFASTA (Pearson and Lipman, 1988) may be used to perform sequence comparisons (Internet Program © 1996, W. R. Pearson and the University of Virginia, "fasta20u63" version 2.0u63, release date December 1996). ALIGN compares entire sequences against one another, while LFASTA compares regions of local similarity. These alignment tools and their respective tutorials are available on the Internet at http://biology.ncsa.uiuc.edu.

Orthologs of the disclosed bispecific fusion proteins are typically characterized by possession of greater than 75% sequence identity counted over the full-length alignment with the amino acid sequence of bispecific fusion protein using ALIGN set to default parameters. Proteins with even greater similarity to the reference sequences will show increasing percentage identities when assessed by this method, such as at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, or at least 98% sequence identity. In addition, sequence identity can be compared over the full length of one or both binding domains of the disclosed fusion proteins. In such an instance, percentage identities will be essentially similar to those discussed for full-length sequence identity.

When significantly less than the entire sequence is being compared for sequence identity, homologs will typically possess at least 80% sequence identity over short windows of 10-20 amino acids, and may possess sequence identities of at least 85%, at least 90%, at least 95%, or at least 99% depending on their similarity to the reference sequence. Sequence identity over such short windows can be determined using LFASTA; methods are described at http://biology.ncsa.uiuc.edu. One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided. The present invention provides not only the peptide homologs that are described above, but also nucleic acid molecules that encode such homologs.

An alternative indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence-

dependent and are different under different environmental parameters. Generally, stringent conditions are selected to be about 5°C to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Conditions for nucleic acid hybridization and calculation of stringencies can be found in Sambrook *et al.* (In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989) and Tijssen (*Laboratory Techniques in Biochemistry and Molecular Biology* Part I, Ch. 2, Elsevier, New York, 1993). Nucleic acid molecules that hybridize under stringent conditions to the disclosed bispecific fusion protein sequences will typically hybridize to a probe based on either the entire fusion protein encoding sequence, an entire binding domain, or other selected portions of the encoding sequence under wash conditions of 0.2 x SSC, 0.1% SDS at 65°C.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that changes in nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequences that each encode substantially the same protein.

Transformed: A transformed cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known in the art.

The present invention provides immortalized osteoblastic cells, including osteoblast precursor cells (OPCs), such as human fetal osteoblastic cells (hFOB) cells, that can be localized in a porous matrix for implantation into bone, to promote the healing of bony defects (such as critical-sized defects) that would heal very slowly, if at all. The OPCs may be transfected with a recombinant bone morphogenetic protein (rBMP), such as recombinant human BMP (rhBMP), for example rhBMP-2, 3, 4, 5, 7 or 9. As used herein, "immortal" or "immortalized" cells refer to a substantially continuous and permanently established cell culture with substantially unlimited cellular viability. That is the cells can be cultured substantially indefinitely. The invention also includes conditionally immortalized cells, which are cells that are mitotic and divide in the presence of a conditionally immortalizing medium, but stop cell division when the conditionally immortalizing component is removed from the medium, but continue to express the proteins

characteristic of OPCs. These cells produce a complement of proteins characteristic of normal human osteoblastic cells and are capable of osteoblastic differentiation.

In some embodiments of this invention, a conditionally immortalized human fetal osteoblastic cell expresses a simian virus 40 (SV40) large T (Tag) or small t (tag) antigen, which is capable of being inactivated. Although the inactivated cells are still viable and express functional proteins characteristic of osteoblasts, they can be put in a state of low proliferation. This inactivation may occur, for example, by placing the SV40 gene under the control of a promoter that relies on the presence of human interferon, or another biological substance. The use of an interferon-dependent promoter is preferred in the disclosed embodiment because interferon is endogenously produced in wounds (such as the osseous defects being treated with the cells), and the levels of interferon taper off as healing occurs. Hence the conditionally immortalized cells are designed to divide rapidly after initial implantation into a wound, but to stop cell division and continue their differentiation into bone-forming osteoblasts as the wound heals.

Although these cells are part of an established "cell line," they are generally non-tumorgenic, i.e. they do not form tumors in mammals. They may be part of a homogeneous population, for example part of a clonal population of a cell line that has been transfected with genes that immortalize, or conditionally immortalize, the cell and code for the expression of a BMP. The term "clonal" refers to a homogenous population of cells derived from a single progenitor cell. The term "transfection" refers to a process by which foreign DNA is introduced into eucaryotic cells and expressed. The foreign DNA is typically included in an expression vector, such as a circular or linearized plasmid vector. In the preparation of a disclosed embodiment of the invention, human osteoprogenitor cells are conditionally immortalized by transfection with the expression vector pMX-1-SV40T. Additionally, the cells can be transfected with a selectable marker gene, such as a gene coding for resistance to an agent normally toxic to the untransformed cells, such as an antibiotic, antineoplastic agent, or a herbicide. In a disclosed embodiment, the cells are transfected with an expression vector that codes for a selection factor such as resistance to neomycin and similar drugs (pMX-1-SV40T-Neo-195), or which expresses a "suicide gene" that enables the transformed cells to be selectively killed.

Disclosed embodiments of the invention have the identifying characteristics of OPC1, which is described in detail in this specification. These cells are clonal, conditionally immortalized osteoblast precursor cells capable of osteoblastic differentiation. They express therapeutically effective amounts of a BMP, such as BMP-2, and/or other factors required for stimulation of bone formation and healing. The cells of the present invention can be prepared from the conditionally immortalized cells of the present invention, and include any replicable expression vector containing a gene coding for an osteogenic BMP. In a disclosed embodiment, the BMP expression vector is a plasmid, for example a plasmid having the identifying characteristics of the KS-hBMP-2, IgSP-NS-hBMP-2, IgSP-KR-hBMP-2, and IgSP-RRRR-hBMP-2 expression vectors.

This osteoblast progenitor cell (OPC) line is responsive to BMP-2 (i.e. cellular differentiation activity is stimulated by exposure to BMP-2) and boosts the bone repair response. Genetically modifying OPCs with a plasmid vector containing a rhBMP-2 gene, and locally introducing the OPCs into a bony defect, allows the OPC to constitutively express BMP to help stimulate and coordinate the cellular processes that repair the defect. Hence a broad variety of osseous defects (such as traumatic bone loss or congenital insufficiency) can be treated using the method of the present invention. The method can also be used in restoring deficient bone mass, congenital malformations, and especially osteopenic vertebrae, which are the most common anatomical site ravaged by osteoporosis. This method amplifies a BMP-responsive cell pool (which is often depleted in the elderly) and augments locally expressed BMP molecules, to counteract decreased vertebral bone mass, diminished bone formation, an imbalance between osteoblasts and osteoclasts, precursor cell decrement, and/or poor bone cell responsiveness. The OPCs that constitutively express BMP enrich local concentrations of these elements that are pivotal to bone regeneration.

BMP SELECTION

BMPs 2-15 are categorized within the transforming growth factor beta superfamily and direct the progression of cells and their organizational format to tissues and organs in the embryo; influence body patterning, limb development, size and number of bones; and modulate post-fetal chondro-osteogenic maintenance (Table 1). An example of a particularly important BMP for the regeneration of bone is BMP-2, which promotes undifferentiated mesenchymal cells into osteoblasts, which lay down the bone. This property has been exploited in preclinical studies with rhBMP-2 to regenerate calvaria; long bone in the rat; rabbit ulna and radius; sheep long bone; the mandible and premaxilla of the dog; and the ulna of the African green monkey. Marden, et al., *J. Biomed. Mater. Res.* 28:1127-1138 (1994); Smith, et al., *J. Controlled Rel.* 36:183-195 (1995); Yasko, et al., *J. Bone Joint Surg.* 74-A:659-671 (1992); Stevenson, et al., *J. Bone Joint Surg.* 76-A:1676-1687 (1994); Cook, et al., J. *Bone Joint Surg.* 76A:827-838 (1994); Hollinger, et al., *J Controlled Rel.* 39:287-304 (1996); Gerhart, et al., *Clin. Orthop. Rel. Res.* 293:317-326 (1993). Furthermore, rhBMP has been applied for spine fusions in dogs and rhesus nonhuman primates, where the rhBMP prompted regeneration of critical-sized defects that ordinarily would not have healed by new bone formation; Boden, et al., *Endocrinol* 137:3401-3407 (1996). In view of the prior use of BMP-2 for these clinical applications, BMP-2 is described in connection with the disclosed embodiment of this invention. However, any of the osteoinductive BMPs can be used in connection with the method of increasing bone formation, for example BMP-3, BMP-4, BMP-5, BMP-7 and BMP-9, and especially BMPs 4 and 9. Other BMPs that are not described as osteogenic in Table 1 may also be used, to the extent that they regulate or stimulate the activity of the directly osteogenic BMPs.

SELECTING OSTEOBLAST PRECURSOR CELLS (OPCs)

The differentiation of osteoblastic cells in culture involves a programmed development sequence. This sequence is characterized by an early proliferative stage during which cells are relatively undifferentiated osteoprogenitor or osteoprecursor cells (OPCs), and later stages which involve the expression of bone cell phenotypic markers and ultimately extracellular matrix mineralization. See, for example, Aronow et al., *J. Cell Physiol.* 143:213 (1989) and Stein et al., *FASEB J.* 4:3111 (1990), which are incorporated by reference. OPCs are cells that differentiate into cells having the phenotypic markers associated with osteoblasts, including expression of osteocalcin (OSC), osteonectin (OSN), osteopontin (OSP), PTH receptor (PTHr), alkaline phosphatase (AP) and procollagen Type I (ProI). OPCs that express the BMP are differentiated into osteoblastic cells by the expressed BMP.

The BMP-mediated strategies to regenerate bone in accordance with the present invention differ from other approaches in that they supply a sufficient quantity of OPCs to restore form and function to bone. The presence of OPCs localized or immobilized in a porous matrix has been found to increase bone formation to a surprising extent. In other embodiments, a sufficient amount of exogenous BMP is provided in the matrix to promote bone deposition. In a particular embodiment, BMP is expressed from the OPC, and the *in situ* availability of BMP from the OPCs minimizes the exogenous dose of rhBMP that must be supplied. In the absence of *in vivo* production from OPCs, milligram quantities of BMP (for example more than about 1.7-2.0 mg doses) are required to produce an osteogenic effect by augmenting a locally responsive cell stock to differentiate into osteoblasts. The *in situ* production of BMP from an OPC (or an osteoblast) allows much smaller doses of the BMP to be delivered, because the BMP is localized by the cells, and delivered in a cellular vehicle that is also intimately involved in the bone deposition process. Therefore, the OPCs will function as a cellular "bioreactor" by synthesizing BMP de novo, allowing the production of a fresh and sustained BMP signal. Administration of BMP from OPCs is especially valuable for the geriatric patient who has a limited number of precursor cells, that may also be functionally challenged.

OPCs can be used from any species, although it is preferred that the OPC selected for use be from the same species as the animal being treated for the bony defect. Hence OPCs from humans, dogs, monkeys, rats, and other species may be used in accordance with this invention. The OPCs may be obtained by conventional techniques, and they are then immortalized (or conditionally immortalized) and localized in a matrix in the bone. Also, the OPCs may be transfected with a gene that expresses a BMP. The following Examples illustrate specific steps in this process.

Especially preferred cells in accordance with the present invention are those osteoprecursor cells that have sufficiently differentiated to commit to an osteogenic lineage. Once this commitment has occurred, the cell will more reliably produce bone in response to BMP

stimulation, instead of becoming a fibrocyte, chondrocyte, adipocyte, or other cells that are the successors of mesenchymal stem cells from which the OPCs of the present invention may be derived. The commitment to bone production is a particular advantage as compared to cells which could produce substantial amounts of fat or fibrosis at the site of the healing bony defect, because significant amounts of those non-bone tissues can interfere with bony union and ultimate healing of the defect.

A specific example of a cell useful with the present invention is the conditionally immortalized cell line OPC1 having the identifying characteristics of ATCC CRL-12471 deposited February 12, 1998, which is an OPC that has committed to the osteogenic lineage, and which can be transfected with a gene to express BMP-2. Commitment to the osteogenic lineage of a cell can be determined by stimulating the cell with a BMP, such as BMP-2, BMP-4, or BMP-9, and observing the development of the osteoblast phenotype by the screening techniques described in Example 2. Cells from the deposited cell line exhibit many preferred characteristics, including the ability to differentiate in response to very low doses of BMP, for example 10 ng/ml concentrations of rhBMP-2, and perhaps even concentrations as low as 5 ng/ml or even 2 ng/ml rhBMP-2. The deposited cell line has also been shown to be able to be passaged for at least P50, for example as much as about P80.

In addition to contacting the BMPs with OPCs, or expressing the BMPs from an OPC, the BMPs can also be contacted with or expressed from more mature osteoblasts (having the phenotype described in Example 2). Like the OPC, an osteoblast is an example of a cell that has committed to an osteogenic lineage, it is just more highly differentiated. The mature osteoblasts do not usually divide, which does not permit the production of a clonal line of dividing cells that can be implanted into a bony defect. However, the BMP gene can be introduced into an OPC which is allowed to mature before implantation into a bony defect, where it is localized and allowed to produce BMP which enhances the healing process. To the extent production of BMP in more mature OPCs and osteoblasts improves bone formation, it is included within the method of the present invention.

Other cells that can be used with the present invention include nucleated blood cells that are present during healing (such as lymphocytes), but which do not stimulate a significant inflammatory response (as would occur with macrophages).

EXAMPLE 1

Establishing Immortalized Human Osteoprogenitor Cell Line OPC-1

The simian virus 40 (SV40) oncogenes, both small t antigen (tag) and large T antigen (Tag), are nuclear phosphoproteins that transform a broad range of cell types. In the present invention, the pMX1-SV40Tag-Neo-195 plasmid DNA (FIG. 3) was utilized in transfection protocols to generate a conditionally immortalized cell line. A "conditionally immortalized" cell

line is one that continues to undergo cell division in a controllable set of circumstances (such as the presence of interferon for an interferon driven promoter), but which can selectively be induced to cease or significantly reduce cell division (for example, by removal from the cellular environment of effective amounts of interferon to drive the promoter).

In this example, the MX-1 promoter directs expression of SV40 large Tag. Transfected cells expressing the pMX-1 DNA exhibit increased proliferation by driving the SV40 Tag in the presence of human A/D interferon (an alpha interferon hybrid constructed from the recombinant human interferons Hu-IFN-αA and Hu-IFN-αD available from PBL, of West Caldwell, NJ). The cells exhibit diminished mitotic activity when interferon is removed. Several laboratories have investigated the establishment of bone cell lines transfected with a gene constitutively expressing the SV40 Tag (Keeting et al., *J. Bone Miner. Res.* 7:127-132 (1992); Evans et al., *J. Orthoped. Res.* 13:317-324 (1995)) while others have utilized a gene coding for a temperature-sensitive mutant, tsA58, of SV40 Tag which conditionally immortalizes the human fetal osteoblastic cell line under permissive conditions; Harris et al., *J. Bone Miner. Res.* 10:178-186 (1995). The SV40 Tag is one of the most effective methods of either constitutively or conditionally immortalizing cell lines.

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Gibco BRL Inc., (Grand Island, NY) unless otherwise noted. Falcon tissue culture plasticware was obtained from Becton Dickson and Co. (Franklin Lakes, NJ). A QuickPrep Micro mRNA Purification Kit was purchased from Pharmacia Biotech, Inc. (Piscataway, NJ), and an Access RT-PCR System was purchased from Promega Inc. (Madison, WI). PCR plasticware was purchased from Perkin Elmer, Inc. (Norwalk, CT). NuSieve 3:1 agarose was obtained from FMC BioProducts (Rockland, ME). The recombinant human bone morphogenetic protein-2 (rhBMP-2) was provided by Genetics Institute, Inc. (Andover, MA) and the methods of production and purification of BMP-2 have been previously described in Wang et al., *Proc. Natl. Acad. Sci.* USA 87:2220-2224 (1990), and Wozney, *Progress in Growth Factors* 1:267-280 (1989).

Fetal tissue of gestational age of approximately 12-13 weeks was obtained under institutionally approved protocols. The tissue was maintained in EBSS supplemented with 10 mM HEPES, pH 7.4, and transported to the laboratory for dissection and isolation. Osteoblasts were derived from fetal human periosteum and femur utilizing a repeated digestion technique involving 0.3% collagenase P (Boeringer-Mannheim, Indianapolis, IN) and 0.25% trypsin, as in Gallagher et al., in *Human Cell Culture Protocols*, Humana Press, pages 233-262 (1996) (which is incorporated by reference). Although three or four repeated digestions may be used, the fourth cellular preparation contains more mature osteoblasts. In contrast, the present method collected the cellular preparations from the first and second digestions, and plated them out in anticipation of isolating and selecting a precursor cell. Cells were plated at 0.25×10^6 in 75 cm^2 tissue culture flasks in alpha MEM with 5% FBS. The remaining tissue pieces were collected, washed with calcium magnesium

free EBSS and digested with 0.25% trypsin-EDTA for 30 min. These cells were also plated as described above. These cultures represent the initial isolation, P0, and once confluent, the cells were subcultured after enzymatic removal with 0.25% trypsin-EDTA to passage 1 (P1).

The early passage bone cells were maintained and expanded to P3, at which time they were transfected by a standard calcium phosphate-mediated methodology (Stratagene®, La Jolla, CA) to incorporate 10 μg of CsCl purified pMX1-SV40T antigen-Neo-195 plasmid DNA into the host cell genome. The plasmid pMX1-SV40T-Neo-195 was fabricated by fusing a 2.3-kb mouse MX-1 promoter to a 2.1-kb SV40 large T fragment in the cloning vector pSP65. A 1.9-kb mouse beta globin 3' untranslated region (3'UTR) was introduced into the plasmid at the BamHI and XbaI sites; the resulting plasmid was named pMX1-SV40T. A 1,518-bp HincII-XmnI fragment containing the neomycin phosphotransferase driven by the SV40 promoter was isolated from pcDNA3 (InVitrogen, San Diego, CA), subcloned into pMX1-SV40T digested with EcoRI, and filled with a Klenow sequence to form the pMX1-SV40T antigen-Neo-195 plasmid DNA (FIG. 3). The P3 bone cells were transfected overnight in a mitogenic serum-free defined medium UltraCULTURE® (BioWhittaker, Inc., Walkersville, MD) containing no antibiotics.

Following the transfection protocol, plates were rinsed with media and placed into fresh alpha MEM/5% FBS overnight. The following day the transfected cells were selected in alpha MEM/5% FBS media supplemented with 0.5 mg/ml G418-sulfate (neomycin analog). The G418 allows the selection of stable transfectants that have incorporated the gene conferring resistance to neomycin toxicity. After a selection period of 10-14 days, the medium was changed to the alpha MEM/5% FBS containing 750 U/mL human A/D interferon and 0.2 mg/ml G418. Clonal lines were obtained by a standard limiting dilution protocol of the polyclonal transfectants and preference was determined by the clonal cell's morphology, growth rate of approximately 3.5-4 doublings per week, and expression of alkaline phosphatase. Mock transfected cells served as a control for the selection.

The osteoblast precursor cell preparations at the time of the initial plating (P0) established an adherent culture with cells exhibiting generally a polygonal, with intermittent fusiform, morphology expressing a faint birefringence surrounding the cells. Within 5-8 days of the P0 plating, the cells grew into a confluent monolayer exhibiting a morphology consistent with other osteogenic cell lines. Control non-transfected cells were maintained and passaged in parallel with the transfected cells to determine the growth rates and limits to propagation. In general, the growth rate of the normal human osteoblast-like cells was reduced as compared to the transfected cell lines (TABLE 3). The non-transformed cells also exhibited a growth rate that significantly diminished after passage 20 (P20), and between P25 and P28 became senescent and ceased propagation.

TABLE 3

OPC clone	Doubling times (days)	APase (control)	APase (10 ng/mlBMP-2)	Mineralize	Passage no.
1	1.8	15.6±1.5	122.4±17.6	+++	10
2	1.9	13.6±2.3	48.6±5.4	++	10
3	1.67	14.5±2.1	55.8±6.6	++	10
4	3.3	*ND	*ND	-	8
5	2.4	*ND	*ND	-	8
6	2.2	14.4±2.4	68.4±5.8	++	10
7	2.9	*ND	*ND	-	8
Nontrans-formed cells	2.4	12.8±1.8	28.8±2.2	+	10

*ND = Not Determined

The cells were subcultured until passage 3 (P3) after which they were transfected with the pMx-1-SV40T-Neo-195 DNA expression vector. Following transfection and selection for 10-14 days in the 0.5 mg/mL G418-sulfate (neomycin analog), 1-2% of the initial plated cells were observed to survive the selection process. In contrast, all of the mock transfected cells died within a 4-7 day period. The stable transfectants were maintained in alpha MEM/5%FBS containing 750 U/mL human A/D interferon and thereafter, the cultures were not maintained under continual selection pressure (i.e. G418-containing medium). Seven clonal lines designated OPC1-OPC7 were obtained by a standard limiting dilution protocol of the polyclonal transfectants in 96 well plates. Three of the clones, OPC4, OPC5, OPC7 were eliminated from additional evaluation as they exhibited undesired morphologic and/or growth characteristics (TABLE 3). In particular, they displayed fusiform morphology (spindle-like, tapering at both ends) and a doubling time greater than or equal to the control non-transformed cell.

The remaining four clones were selected for additional expansion and characterization. Morphologically, these clonal cells generally exhibited a polygonal morphology with the extension of short dendritic processes at low density, which when grown to a confluent monolayer possessed an epithelial morphology that did not exhibit extensive hyperconfluence, thus

cell growth was somewhat contact inhibited. Growth curve analysis for the 4 clones, OPC1, OPC2, OPC3 & OPC6 approximated 3.5-4.2 doublings per week (population doubling time of 40-49 hrs) and the clone designated OPC1 was selected as the lead candidate based on the highest level of alkaline phosphatase (APase) expression, but especially the ability of the OPC1 line to significantly up-regulate the APase activity to low dose rhBMP-2, 10 ng/ml, as assessed at day 9. OPC1 can be maintained in a nutrient rich medium such as alpha MEM with 5% FBS, but it also demonstrates an ability to survive in conditions that are nutrient poor (a serum level of approximately 0.6-0.9% v/v). This ability to survive in a nutrient poor environment is a particular advantage of OPC1 that renders it especially useful in the method of the present invention in which the cell is implanted *in situ* into a nutrient poor osseous defect.

Cryovials containing 5 x 10^6 cells of the OPC1 line maintained in antibiotic and antimycotic-free tissue culture medium for at least 3 passages were packaged and sent to ViroMED Laboratories (Minneapolis, MN) to test for the presence of Cytomegalovirus, Hepatitis B, Hepatitis C, HIV-1, HIV-2, and HTLV I/II. Conditioned medium was also collected and shipped to Microbiological Associates, Inc. (Rockville, MD) for assessing sterility. Lastly, mycoplasma detection was performed utilizing the CELLshipper™ kit as described by BIONIQUE® Testing Laboratories, Inc. (Saranac Lake, NY). The OPC1 line was determined to be free of these pathogens.

EXAMPLE 2

Screening Methods to Determine if Cell has OPC Phenotype

Confirmation of an osteoblast/pre-osteoblast phenotype was performed at various passages (P10, P20, P30) with OPC1. The APase enzyme activity was quantitatively measured by the method of Lowry et al., *J. Biol. Chem.* 207:19-37 (1954) in cultures at days 4, 9 and 16 following an initial seeding of 25K cells/well in a 6 well plate (Falcon) overnight with a base medium of alpha MEM/5% FBS (GIBCO). The osteoblast/pre-osteoblast/OPC phenotype primarily requires the identification of a marker for osteocalcin, and preferably at least two other markers selected from the group of alkaline phosphatase, mineralization, osteonectin, osteopontin, PTH-receptor, and procollagen.

Alkaline Phosphatase Activity

The wells with the OPCs contained the base medium of alpha MEM/5% FBS (GIBCO) overnight after their initial seeding. On the following day, the base medium provided a negative control (1) and the additional groups included: (2) base medium supplemented with 10 ng/ml of rhBMP-2; (3) base medium supplemented with 50 ng/ml of rhBMP-2; (4) base medium supplemented with 100 ng/ml of rhBMP-2; (5) base medium supplemented with the osteogenic supplement (OS) 10 mM beta-glycerophosphate, 10-7 M Dexamethasone, 50 μg/ml of ascorbic acid

phosphate (Wako Chemical, Osaka, Japan) and (6) base medium supplemented with OS plus 50 ng/ml rhBMP-2. Alkaline phosphatase enzyme activity was measured in triplicate cultures after rinsing the wells with calcium magnesium-free EBSS, collecting the cells by scraping and incubating 50K per well in a 96 well plate with 5 mM p-nitrophenyl phosphate in 50 mM glycine and 1 mM $MgCl_2$ at 37° C for 5 to 20 min. Enzyme activity was calculated after measuring the absorbance of the p-nitrophenol product formed at 405 nm on a microplate reader (MRX, Dynatech Labs., Chantilly, VA) and compared to serially diluted standards. Enzyme activity is expressed as ng of p-nitrophenol/min/50K cells. In addition, APase histochemistry was performed on cultures at P30 according to standard protocols described in Sigma Kit #85. However, in this series of experiments, group (4) from above (base medium supplemented with 100 ng/ml of rhBMP-2), was replaced with base medium supplemented with 50 ng/ml of bFGF, to evaluate mitogenicity and/or the ability to influence programmed osteogenic differentiation.

At P20, with the exception of the control, the OPCs exhibited a statistically significant increase in the APase enzyme activity in all medium conditions at 4, 9 and 16 days after the initial seeding period. A striking up-regulation of APase enzyme activity was observed at 4 days in an osteogenic supplement (OS) group (base medium supplemented with 10 mM beta-glycerophosphate, 10^{-7} dexamethasone, 50 μg/ml of ascorbic acid phosphate (Wako Chemical, Osaka, Japan)) and 50 + OS (base medium supplemented with OS and 50 ng/ml rhBMP-2) group. Four days was also the time at which the wells approached confluence. These groups exhibited activities of 63.2 ± 10.2 and 262.3±14.8 ng p-nitrophenol/min/50K cells, respectively, as compared to 9.6 ± 2.4 for the control. By day 9, peak APase enzyme activities in ng p-nitrophenol/min/50K cells were observed: control=16.6 ± 2.6; 10 ng BMP=127.8 ± 19.5; 50 ng BMP=225.5 ± 22.8; 100 ng BMP= 292.2 ± 24.4; OS=310.1 ± 19.2; 50 + OS=407.8 ± 19.5. Similar observations were noted for the OPC1 line at P10 and P30. APase histochemistry revealed staining that was consistent with the enzyme activity data.

Mineralization

The OPC1 line was evaluated for the cells' ability to mineralize the extracellular matrix which they produce 7-10 days following confluence in the base medium, but especially following maintenance in the base medium supplemented osteogenic supplement (OS). The extracellular calcium content was quantitatively measured by scraping twice rinsed with PBS cell layers and exposing the cells to 0.1 N HCl. The cells were extracted by shaking for 4 hours at 4°C, collecting the cells by centrifugation, and using the supernatant for calcium determination according to the manufacturer's protocol in Sigma Kit #587. Absorbance of the sample was measured on the multiplate reader (MRX, Dynatech Labs) at 570 nm at 5-10 min after the addition of reagents. Total calcium was calculated from standard solutions prepared in parallel and expressed as μg/well.

A histochemical analysis of mineralization was also evaluated utilizing the staining procedure of von Kossa. Postconfluent cells were fixed in 1% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 1 hr, rinsed with PBS and treated with 5% (w/v) silver nitrate in the dark for 15 min. The cells were then rinsed thoroughly with distilled water, subjected to ultraviolet light for 5-7 min, treated with sodium carbonate/formaldehyde solution for 2 minutes and finally with Farmer's reducer for 1 min.

The OPC1 line has been characterized quantitatively for extracellular calcium deposition during the formation of the mineralized nodules at P20. All conditions were negative for calcium content at day 4, while the groups with osteogenic supplement (± 50 ng/ml rhBMP-2) have exhibited a significant increase in the quantity of extracellular matrix deposition at day 9. By day 16, all of the groups have exhibited a significant increase in the deposition of extracellular calcium, while the groups maintained in the osteogenic supplement have deposited nearly 20 μg of calcium in a 6 well plate.

The von Kossa stained specimens were detected under light microscopy by day 4-5 postconfluency and were extensive by days 7-10 in the treatment groups maintained in the 50 ng rhBMP-2 + OS. Once the cells reached confluency, a small number of mineralized nodules were visible in the cells maintained in base medium of alphaMEM/5% FBS at day 9 following the initial seeding. However, in groups maintained in the BMP groups ± the OS, and especially in the 50 ng rhBMP-2 + OS cultures, the number of mineralized nodules was markedly greater than controls. The formation of mineralized nodules in the cells maintained in base medium without beta-glycerophosphate or dexamethasone is consistent with a previous report for immortalized human fetal osteoblastic cells (Harris, et al., *J. Bone Miner. Res.* 10:178-186 (1995)) but is otherwise unusual for osteoblastic cell lines.

Osteocalcin

The osteocalcin level in conditioned medium was determined by an EIA kit for intact osteocalcin (Biomedical Technologies, Inc., Stoughton, MA). Pre- and post-confluent OPC1 cells in 6 well multiwell plates (characterized in triplicate) were maintained in 1 ml of the serum-free medium UltraCULTURE® with the various supplements included as previously described in the measurement of APase. Samples were collected after 48 hrs and a 50 μl sample was innoculated onto the microtiter plate and assayed according to the manufacturer's protocol. Data are expressed as ng/ml and the limit of detection for the EIA is 0.1 ng/ml.

Intact osteocalcin (ng/ml/24 hr) was measured in the tissue culture medium under the following conditions: control= 1.1 ± 0.3; 10 ng BMP=*1.7 ± 0.3; 50 ng BMP=*1.8 ± 0.3; 50 ng bFGF= 0.8 ± 0.2; OS=* 2.1 ± 0.5; 50 + OS= *8.6 ± 2.7. A significant increase (*$p < 0.05$) as compared to the control group was observed in all the treatment groups except the bFGF group. The lysate values from approximately 1 x 10^6 OPC1 cells maintained in the control

medium was negligible (below the limit of detection), further indicating that the osteocalcin measured in the present study was intact and secreted *de novo* from the OPC1 line.

Reverse Transcriptase Analysis

Established Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) qualitative analysis techniques were used to detect (by PCR phenotyping) the presence of osteocalcin (OSC), osteonectin (OSN), osteopontin (OSP), PTH receptor (PTHr), alkaline phosphatase (ALP) and procollagen Type I (ProI). Rickard, et al., *J. Bone Miner. Res.* 11:312-324 (1996); Bilbe et al., *Bone* 19:437-445 (1996). The oligonucleotide RT-PCR primer sequences are listed in TABLE 4 and were purchased from GIBCO BRL.

TABLE 4

Reverse Transcriptase-PCR Primers for Phenotype Analysis

RT-PCR Primer Set	Sequence	Product Length
Osteocalcin (SEQ. ID. Nos. 1 and 2)	5'-ctggccctgactgcattctgc-3' 5'-aacggtggtgccatagatgcg-3'	258bp
Osteonectin (SEQ. ID. Nos. 3 and 4)	5'-gatgaggacaacaaccttctgac-3' 5'-ttagatcacaagatccttgtcgat-3'	369bp
Osteopontin (SEQ. ID. Nos. 5 and 6)	5'-aaatacccagatgctgtggc-3' 5'-aaccacactatcacctcggc-3'	348bp
PTH-Receptor (SEQ. ID. Nos. 7 and 8)	5'-aggaacagatcttcctgctgca-3' 5'-tgcatgtggatgtagttgcgcgt-3'	571bp
Alkaline Phosphatase (SEQ. ID. Nos. 9, 10)	5'-gcgaacgtatttctccagacccag-3' 5'-ttccaaacaggagagtcgcttcaa-3'	367bp
Procollagen I (SEQ. ID. Nos. 11, 12)	5'-tgacgagaccaagaactg-3' 5'-ccaaagtcaccaaacctacc-3'	599bp

The procedure involves 3-5 x 10^6 OPCs pelleted at 12,000 RPM in a microfuge (Eppendorf 5412, Brinkman Instruments, Inc. Westbury, NY) and either used immediately or frozen at -80 °C for storage. Then mRNA was isolated using the QuickPrep Micro mRNA purification kit (Pharmacia Biotech, Inc.) according to the manufacturer's specifications resulting in a 200 μl final mRNA elution volume. The mRNA concentrations were determined by

spectrophotometric absorbance using A_{260} x 40 μg/μl. mRNA concentrations of the elution volumes were determined to be in the range of 50-160 μg/μl. The cDNA was synthesized from 50 μg of the mRNA according to the Access RT-PCR System (Promega, Inc., Madison, WI). Aliquots of the total cDNA were amplified in each PCR with 2.5 U of *Taq* polymerase (Promega, Inc.) and amplifications were performed in a GeneAmp 2400 thermal cycler (Perkin-Elmer, Inc., Norwalk, CT) for 30 cycles after an initial 30 sec denaturation at 94°C, annealed for 2 min at 55°C, and extended for 2 min at 72°C. The amplification reaction products were resolved by 2.5% NuSieve agarose/TBE gels (FMC BioProducts, Rockland, ME) electrophoresed at 85 mV for 90 min and visualized by ethidium bromide. Base ladders of 50 bp and 100 bp (Boehringer Mannheim, Inc.) provided standards.

The OPC1 line expressed abundant mRNA generated from 30 cycles of RT-PCR for the presence of osteocalcin (OSC), osteonectin (OSN), osteopontin (OSP), PTH receptor (PTHr), alkaline phosphatase (AP) and procollagen Type I (ProI). The representative RT-PCR products (bands) were resolved by agarose gel electrophoresis and correspond to base pair product lengths of 258, 369, 348, 571, 367 and 599 for OSC, OSN, OSP, PTHr, AP and ProI, respectively, as outlined in TABLE 4.

The levels of expression of these positive markers appears to be influenced by the conditions in which the OPC1 line are maintained. For example, osteocalcin and PTH receptor messages (markers corresponding to the late phase of osteoblast differentiation) appear to have greater intensity in the cells maintained in the 50+ OS medium condition than the base medium alone. Human-derived glioblasts have provided a control cell type and are negative for OSC, OSN, OSP and PTHr.

This example therefore demonstrates and characterizes a new human fetal osteoprecursor cell line (OPC1) which is immortalized with a gene coding for the SV40 large T antigen (Tag). The incorporation of the DNA plasmid into the primary cultures drives a gene, the MX-1 promoter, to conditionally express SV40 large T antigen when activated by human A/D interferon. However, when the OPC1 line is maintained in nutrient-rich tissue culture medium, i.e., alpha MEM containing 5% (v/v) FBS, no difference in the rate of cell proliferation is observed either in the presence or absence of human A/D interferon. The Tag is therefore expressed constituitively. When the OPC1 reaches confluence in standard two-dimensional tissue culture plastic, the cells exhibit contact inhibition, with a concurrent down regulation of immunopositive nuclear Tag staining. Hence the OPC1 line does not exhibit tumorigenic characteristics. Additionally, these cells have been repeatedly frozen and thawed and continue to maintain consistent levels of the osteogenic phenotypic markers, further indicating that a successful derivation of an osteoprecursor clonal cell line was accomplished.

Numerous phenotypic markers establish the stability of expression in the OPC1 line in association with osteoblastic differentiation. These include alkaline phosphatase expression,

the ability to mineralize, measurement of intact osteocalcin, and mRNA expression of osteocalcin (OSC), osteonectin (OSN), osteopontin (OSP), PTH receptor (PTHr), alkaline phosphatase (AP) and procollagen Type I (ProI). The present studies indicate that postconfluent cultures of the OPC1 line express high levels of these osteoblastic-associated markers at passage 10 (P10), P20 and P30.

In the present studies, rhBMP-2 elicits a stimulatory effect on APase activity in the OPC1 line *in vitro*. However, rhBMP-2 has no stimulatory effect on differentiated osteoblasts obtained from human iliac bone or a more differentiated rat-derived osteoblast cell, ROB-C20. Yamaguchi et al., *J. Cell Biol.* 113:681-687 (1991). Thus, based on the OPC1 line's capacity to generate programmed osteoblastic differentiation in the presence of low dose rhBMP-2 (10 ng/ml), the OPC1 line represents a homogeneous osteoprecursor cell line. The absence of any fibroblastic or adipocytic activity also indicates that the OPC has sufficiently differentiated to commit to an osteogenic lineage. The ability to demonstrate programmed differentiation at a dose of 10 ng/ml rhBMP-2 is markedly lower than described for the mouse-derived MC3T3-E1 (Takuwa et al., *Biochem. Biophys. Res. Commun.* 174:96-101 (1991)), a rat-derived "potential" osteoblast precursor cell line (Yamaguchi et al. (1991)), and differentiated osteoblasts from human iliac bone (Kim et al., *J. Biomed. Mater. Res.* 35:279-285 (1997)).

The OPC1 (and other cells that can be obtained by the methods of the present invention) provide a consistent and reproducible culture system to provide a biomimetic for endogenous human osteoprecursor cells.

EXAMPLE 3

Transfection with Suicide Gene

This example discloses an alternative embodiment of the invention in which a SV40 plasmid incorporates a suicide gene that enables the OPCs incorporating the plasmid to be selectively destroyed. Some of the early passage bone cells are transfected with the pMX1-SV40-Tt antigen construct with a TK-neomycin gene for selection. This DNA construct contains both the large T and small t antigen. Ten micrograms of CsCl purified pMX1-SV40T t-antigen-Neo-431 DNA (p431) are introduced into the pre-osteoblast cells at P3 using GIBCO BRL's LIPOFECTAMINE™ PLUS mammalian transfection kit for 6 hrs suspended in a mitogenic serum-free defined medium UltraCULTURE® from BioWhittaker, Inc. containing no antibiotics. Following the transfection protocol, plates are rinsed with media and placed into fresh alpha MEM/5% FBS overnight. The following day the transfected cells are exposed to alpha MEM/5% FBS media supplemented with 0.5 mg/ml G418-sulfate (GIBCO BRL-neomycin analog). The G418 allows the selection of stable transfectants that have incorporated the gene conferring resistance to neomycin toxicity.

After a selection period of 10-14 days, the medium is changed to the alpha MEM/5%FBS containing 750 U/mL human A/D interferon and 0.2 mg/ml G418. Clonal lines are

obtained by a standard limiting dilution protocol of the polyclonal transfectants and preference is based on the clonal cell's morphology, growth rate of approximately 3.5-4 doublings per week, and abundant expression of alkaline phosphatase. The MX-1 promoter conditionally activates the expression of SV40-large T and/or small t antigens when stimulated by human A/D interferon. The conditional immortalizing gene can be reversible, i.e., upon removal of the stimulating condition (interferon production), the preferred cell line will exit the cell cycle, commit to a differentiated phenotype, and exist in a stable, non-mitotic state. The small t antigen generates cell lines that exhibit a truly conditional immortalizing state. The TK-neomycin gene offers the additional advantage of providing a suicide gene in the construct for safety purposes which can destroy the cells in the presence of the antibiotic ganciclovir, valganciclorvir, acyclovir, or related compounds if desired or necessary.

A pre-osteoblast cell can also be transfected with hCNTF-pNUT-DNT. A plasmid expression vector containing the hCNTF gene was constructed by introducing a linker generating a *SmaI* site introduced at +600 of the mouse metallothionein-1 (MT-1) promoter. This *SmaI* site was fused to a Klenow-filled XbaI site at the 5' end of a approximately 150 base pair (bp) human immunoglobulin region containing the hCNTF cDNA. The hCNTF gene was obtained by PCR amplification of human DNA with primers that include an EcoRI site at the position of the natural hCNTF initiation codon and *BglII* site 7 bp 3 of its termination codon. A 325 bp PavI fragment containing the poly-adenylation signal sequence has been modified such that a BamHI site can be added to the 5' end and a NotI site added to the 3' end. This fragment was cloned into the *BglII*-NotI sites on the 3' end of the hCNTF gene. The entire 2854 bp MT-1/Ig/hCNTF-2/hGH KpnI-NotI fragment was then inserted between the Kpn and NotI sites of a pNUT vector in which the EcoRI site was converted to a NotI site by inserting a linker into a Klenow-filled EcoRI site. A 2 kb PvuII fragment containing the herpes simplex virus-thymidine kinase (HSV-tk) gene was cloned into the EcoRV site of the Bluescript and the XhoI site was converted to NotI such that the NotI fragment containing the HSV-TK gene was isolated and inserted into the NotI site to generate the current plasmid expression vector. This vector (shown in FIG. 7) has been utilized to transfect the OPCs to produce hCNTF and has been used to transfect C2C12 myoblasts and BHK cells.

Plasmids have also been used which utilize the MX-1-KS-v-myc+NEO^R and the CMV-KS-v-myc+NEO^R. The plasmid with the MX-1 promoter conditionally activates the v-myc oncogene in the presence of human interferon. The second plasmid has the KS-v-myc gene under control of the CMV promoter, to result in efficient levels of expression. Other alternatives include conditional immortalizing and growth factor/bone differentiating factor plasmids that are under the activation of bone-specific promoters such as osteocalcin.

EXAMPLE 4

Determining Transgene Expression

Immunostaining for the presence of positive large T antigen (Tag) expression is performed on the OPC line that has been maintained in medium that drives the promoter for Tag (human A/D interferon in the conditionally immortalized example), and in cultures without the stimulus. The Tag monoclonal antibody is available from CytoTherapeutics, Inc. (Lincoln, RI), and the staining protocol follows standard immunoperoxidase techniques utilizing a Vectastain Elite® ABC Mouse Kit. The SCT-1/hNGF cell line is utilized as the positive control for the immunopositive nuclear Tag; Schinstine et al., *Cell Trans.* 4:93-102 (1995).

Immunostaining revealed positive nuclear staining for the presence of positive large T antigen (Tag) expression in the OPC1 line maintained in medium with human A/D interferon, that drives the promoter for Tag. In the absence of the stimulating agent, some of the OPCs still retain a positive nuclear immunostaining for the Tag antibody (5-8%), suggesting that the large T antigen may be constituitively expressed in some of the OPC1 line, rendering these cells immortal and not retaining the capacity for reversal. However, in preferred embodiments, the OPC cells selected for use in the method of the present invention do not constitutively express the large T antigen. The SCT-1/hNGF cell line exhibited immunopositive nuclear Tag as a positive control.

In summary, the OPC1 line is a contaminant-free human-derived immortalized osteoprecursor cell line that appears to exhibit contact inhibition and undergoes programmed osteogenic differentiation. This cell line has exhibited a stable incorporation of the SV40-T antigen transgene, without continual selection pressure, that does not appear negatively to impact the growth, maintenance or differentiation genome of the host cell. The OPC1 line can be maintained for greater than 80 passages, and does not exhibit growth crisis and senescence observed in the non-transformed parent cell line. The OPC1 has also been utilized to stably incorporate a second transgene to produce and secrete a growth or differentiation factor. The osteoprecursor cell line also provides a sensitive *in vitro* cell culture system to evaluate bone development, cell/biomaterial interactions, and screen for putative bone differentiating factors.

Construction of hBMP-2 Expression Vectors

A variety of hBMP expression vectors can be used to introduce BMP genes into the OPC cells of the present invention. The following examples (5-10) illustrate some specific examples of varying approaches to constructing hBMP-2 expression vectors for transfection into the OPC cells, and serves as an example for other hBMPs.

EXAMPLE 5

Construction of the KS-hBMP-2 Expression Vectors pcDNA3.1(+)-KS-hBMP2-508 and pPI-DN-KS-hBMP2-512

Total RNA from the OPC1 line is prepared using the guanidinium thiocyanate-based TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). Five hundred ng of the OPC1 line total RNA is reverse transcribed at 42°C for 30 minutes in a 20 ml reaction volume containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 4 mM of each dNTP, 5 mM $MgCl_2$, 1.25 mM oligo(dT) 15-mer, 1.25 mM random hexamers, 31 units of RNase Guard RNase Inhibitor (Pharmacia, Sweden) and 200 units of SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg, MD). Two microliters of the above reverse transcribed cDNA is added to a 25 ml PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 800 nM of each dNTP, 2 mM $MgCl_2$, 400 nM of primers ohBMP2-597 and ohBMP2-598, and 2.5 units of Thermus aquatics (Taq) DNA polymerase (Boehringer Mannheim, Germany). The primer ohBMP2-597 has the synthetic HindIII restriction site and the consensus ribosome binding site (referred to as Kozak Sequence, KS, hereafter) at the 5' end whereas ohBMP2-598 has BamHI at the 5' end.

The PCR reaction mixture is subjected to 30 amplification cycles consisting of: denaturation, 94°C 30 seconds (first cycle 2 minutes); annealing, 50°C 1 minute; and extension, 72°C 3.5 minutes (last cycle 5 minutes). The 1215-bp hBMP-2 PCR product is digested with restriction endonucleases BamHI and HindIII and resolved on an 1% Trivie agarose gel. The 1215-bp HindIII/BamHI DNA fragment is excised and purified using the Spin-X DNA purification kit (Corning Costart Corporation, Cambridge, MA). The pcDNA3.1(+) expression vector is also digested with BamHI and HindIII and purified from 1% agarose using the Spin-X DNA purification kit (Corning Costart Corporation, Cambridge, MA). The ligation mixture is transformed into *E. coli* DH5α (Gibco BRL, Gaithersburg, MD). A cracking gel procedure (Promega Protocols and Applications Guide, 1991) is used to screen out the positive sub-clones.

The identity of the correct clones will be further verified by BamHI/HindIII double digestion. The positive sub-clone for the full-length hBMP-2 in pcDNA3.1(+) is named pcDNA3.1(+)-hBMP-2-508. The nucleotide sequence of the full-length hBMP-2 clone is determined by the dideoxynucleotide sequence determination using the SequaTherm kit (Epicentre Technologies, Madison, WI) for the automated DNA Sequencer. Subsequently, the full-length KS-hBMP-2 insert is subcloned out of pcDNA3.1(+)-hBMP-2-508 by NheI/NotI digestions and directionally cloned into the pPI-DN expression vector resulting in pPI-DN-KS-hBMP2-512.

EXAMPLE 6

Construction of the IgSP-NS-hBMP-2 expression vectors: pcDNA3.1(+)-IgSP-NS-hBMP2-509 and pPI-DN-IgSP-NS-hBMP2-513

Recombinant PCR methodology is used to generate the IgSP-NS-hBMP-2 fusion gene. Oligonucleotides oIgSP-221 and ohBMP2-601 are specific for the IgG signal peptide sequence (IgSP) and the mature hBMP-2 sequence, respectively, and contain synthetic HindIII and BamHI restriction sites at the 5' end, respectively. Oligonucleotides ohBMP2-599 and ohBMP2-600 are complementary to each other. Furthermore, oligonucleotide ohBMP2-600 has its 5' 14 nucleotides identical to the IgSP sequence and its 3' 16 nucleotides identical to the mature hBMP-2 sequence; and vice versa for ohBMP2-599. Two first PCR reactions are carried out using oligonucleotide pairs oIgSP-221/ohBMP2-599 and ohBMP2-600/ohBMP2-601 on templates pNUT-hCNTF-TK and pcDNA3.1(+)-KS-hBMP-2-508 plasmids, respectively. One hundred ng of template DNA is added to a 50 ml PCR reaction mixture containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 800 nM of each dNTP, 2 mM $MgCl_2$, 400 nM of primers #1 and #2, and 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Germany).

The PCR reaction mixtures are subjected to 30 amplification cycles consisting of: denaturation, 94°C for 30 seconds; annealing, 50°C 30 seconds; and extension, 72°C 30 seconds. The PCR products are resolved on 1% TrivieGel (TrivieGen). Two agarose plugs containing each one of the first PCR products are transferred to a tube containing 50 ml of PCR reaction mixtures identical to the one described above with the exception that the oligonucleotides oIgSP-221 and ohBMP2-601 are used. The second PCR reaction is subjected to 30 amplification cycles consisting of: denaturation, 94°C for 30 seconds (first cycle 2 minutes); annealing, 60°C 30 seconds (second to fourth cycles 37°C 2 minutes); and extension, 72°C 30 seconds (last cycle 2 minutes). The 535 bp IgSP-mature hBMP-2 fusion PCR product and the cloning vectors pcDNA3.1(+) are digested with BamHI and HindIII restriction enzymes and subsequently purified from 1% Trivie and agarose gels, respectively, using the Spin-X DNA purification kit (Corning Costart Corporation, Cambridge, MA). The ligation mixture is transformed into *E. coli* DH5α (Gibco BRL, Gaithersburg, MD). A cracking gel procedure (Promega Protocols and Applications Guide, 1991) is used to screen out the positive sub-clones.

The identity of the correct clones will be further verified by BamHI/HindIII double digestion. The positive sub-clones for the IgSP-NS-hBMP-2 is named pcDNA3.1(+)-IgSP-NS-hBMP2-509. The nucleotide sequence of the IgSP-NS-hBMP-2 clone is determined by the didexoynucleotide sequence determination using the SequaTherm kit (Epicentre Technologies, Madison, WI) for the automated DNA Sequencer. Subsequently, the IgSP-NS-Hbmp-2 insert is subcloned out of pcDNA3.1(+)-IgSP-NS-hBMP2-509 by NheI/NotI digestions and directionally cloned into the pPI-DN expression vector resulting in pPI-DN-IgSP-NS-hBMP2-513.

EXAMPLE 7

Construction of the IgSP-KR-hBMP-2 expression vectors: pcDNA3.1(+)-IgSP-KR-hBMP2-510 and pPI-DN-IgSP-KR-hBMP2-514

Recombinant PCR methodology is used to generate the IgSP-KR-hBMP-2 fusion gene. Oligonucleotides oIgSP-221 and ohBMP2-601 are specific for the IgG signal peptide sequence (IgSP) and the mature hBMP-2 sequence, respectively, and contain synthetic HindIII and BamHI restriction sites at the 5' end, respectively. Oligonucleotides ohBMP2-602 and ohBMP2-603 are complementary to each other. Furthermore, oligonucleotide ohBMP2-603 has its 5' 14 nucleotides identical to the IgSP sequence and its 3' 16 nucleotides identical to the mature hBMP-2 sequence; and vice versa for ohBMP2-602. Two first PCR reactions are carried out using oligonucleotide pairs oIgSP-221/ohBMP2-602 and ohBMP2-603/ohBMP2-601 on templates pNUT-hCNTF-TK and pcDNA3.1(+)-KS-hBMP-2-508 plasmids, respectively. One hundred ng of template DNA is added to a 50 ml PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 800 nM of each dNTP, 2 mM $MgCl_2$, 400 nM of primers #1 and #2, and 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Germany).

The PCR reaction mixtures are subjected to 30 amplification cycles consisting of: denaturation, 94°C for 30 seconds; annealing, 50°C 30 seconds; and extension, 72°C 30 seconds. The PCR products are resolved on 1% TrivieGel (TrivieGen). Two agarose plugs containing each one of the first PCR products are transferred to a tube containing 50 ml of PCR reaction mixtures identical to the one described above with the exception that the oligonucleotides oIgSP-221 and ohBMP2-601 are used. The second PCR reaction is subjected to 30 amplification cycles consisting of: denaturation, 94°C for 30 seconds (first cycle 2 minutes); annealing, 60°C 30 seconds (second to fourth cycles 37°C 2 minutes); and extension, 72°C 30 seconds (last cycle 2 minutes). The 541 bp IgSP-KR-hBMP-2 fusion PCR product and the cloning vectors pcDNA3.1(+) are digested with BamHI and HindIII restriction enzymes and subsequently purified from 1% Trivie and agarose gels, respectively, using the Spin-X DNA purification kit (Corning Costart Corporation, Cambridge, MA). The ligation mixture is transformed into *E. coli* DH5α (Gibco BRL, Gaithersburg, MD). A cracking gel procedure (Promega Protocols and Applications Guide, 1991) is used to screen out the positive sub-clones.

The identity of the correct clones will be further verified by BamHI/HindIII double digestion. The positive sub-clones for the IgSP-KR-hBMP-2 are named pcDNA3.1(+)-IgSP-KR-hBMP2-510. The nucleotide sequence of the IgSP-KR-hBMP-2 clone is determined by the didexoynucleotide sequence determination using the SequaTherm kit (Epicentre Technologies, Madison, WI) for the automated DNA Sequencer. Subsequently, the IgSP-NS-hBMP-2 insert is subcloned out of pcDNA3.1(+)-IgSP-KR-hBMP2-510 by NheI/NotI digestions and directionally cloned into the pPI-DN expression vector resulting in pPI-DN-IgSP-KR-hBMP2-514.

EXAMPLE 8

Construction of the IgSP-RRRR-hBMP-2 expression vectors: pcDNA3.1(+)-IgSP-RRRR-hBMP2-511 and pPI-DN-IgSP-RRRR-hBMP2-515

Recombinant PCR methodology is used to generate the IgSP-RRRR-hBMP-2 fusion gene. Oligonucleotides oIgSP-221 and ohBMP2-601 are specific for the IgG signal peptide sequence (IgSP) and the mature hBMP-2 sequence, respectively, and contain synthetic HindIII and BamHI restriction sites at the 5' end, respectively. Oligonucleotides ohBMP2-604 and ohBMP2-605 are complementary to each other. Furthermore, oligonucleotide ohBMP2-605 has its 5' 14 nucleotides identical to the IgSP sequence and its 3' 16 nucleotides identical to the mature hBMP-2 sequence; and vice versa for ohBMP2-604. Two first PCR reactions are carried out using oligonucleotide pairs oIgSP-221/ohBMP2-604 and ohBMP2-605/ohBMP2-601 on templates pNUT-hCNTF-TK and pcDNA3.1(+)-KS-hBMP-2-508 plasmids, respectively. One hundred ng of template DNA is added to a 50 ml PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 800 nM of each dNTP, 2 mM $MgCl_2$, 400 nM of primers #1 and #2, and 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Germany).

The PCR reaction mixtures are subjected to 30 amplification cycles consisting of: denaturation, 94°C for 30 seconds; annealing, 50°C 30 seconds; and extension, 72°C 30 seconds. The PCR products are resolved on 1% TrivieGel (TrivieGen). Two agarose plugs containing each one of the first PCR products are transferred to a tube containing 50 ml of PCR reaction mixtures identical to the one described above with the exception that the oligonucleotides oIgSP-221 and ohBMP2-601 are used. The second PCR reaction is subjected to 30 amplification cycles consisting of: denaturation, 94°C for 30 seconds (first cycle 2 minutes); annealing, 60°C for 30 seconds (second to fourth cycles 37°C, 2 minutes); and extension, 72°C for 30 seconds (last cycle 2 minutes). The 547 bp IgSP-RRRR-hBMP-2 fusion PCR product and the cloning vectors pcDNA3.1(+) are digested with BamHI and HindIII restriction enzymes and subsequently purified from 1% Trivie and agarose gels, respectively, using the Spinx-X DNA purification kit (Corning Costart Corporation, Cambridge, MA). The ligation mixture is transformed into *E. coli* DH5α (Gibco BRL, Gaithersburg, MD). A cracking gel procedure (Promega Protocols and Applications Guide, 1991) is used to screen out the positive sub-clones.

The identity of the correct clones is further verified by BamHI/HindIII double digestion. The positive sub-clones for the IgSP-RRRR-hBMP-2 are named pcDNA3.1(+)-IgSP-RRRR-hBMP2-511. The nucleotide sequence of the IgSP-RRRR-hBMP-2 clone is determined by the didexoynucleotide sequence determination using the SequaTherm kit (Epicentre Technologies, Madison, WI) for the automated DNA Sequencer. Subsequently, the IgSP-RRRR-hBMP-2 insert is subcloned out of pcDNA3.1(+)-IgSP-RRRR-hBMP2-511 by NheI/NotI digestions and directionally cloned into the pPI-DN expression vector resulting in pPI-DN-IgSP-RRRR-hBMP2-515.

The nucleotide sequences of the plasmid vectors described in this example are shown in TABLE 5.

TABLE 5

Nucleotide Sequences of the KS-hBMP-2, IgSP-NS-hBMP-2, IgSP-KR-hBMP-2, and IgSP-RRRR-hBMP-2 Genes

KS-hBMP-2.seq (SEQ. ID. No. 13)

CCCaagcttCGCCACCatggtggccgggacccgctgtcttctagcgttgctgcttccccaggtcctcctgggcggcgcggctggcctcgtt
ccggagctgggccgcaggaagttcgcggcggcgtcgtcgggccgcccctcatcccagccctctgacgaggtcctgagcgagttcgagttgcgg
ctgctcagcatgttcggcctgaaacagagacccacccccagcagggacgccgtggtgccccccctacatgctagacctgtatcgcaggcactcag
gtcagccgggctcacccgccccagaccaccggttggagagggcagccagccgagccaacactgtgcgcagcttccaccatgaagaatctttgg
aagaactaccagaaacgagtgggaaaacaacccggagattcttctttaatttaagttctatccccacggaggagtttatcacctcagcagagcttcag
gttttccgagaacagatgcaagatgctttaggaaacaatagcagtttccatcaccgaattaatatttatgaaatcataaaacctgcaacagccaactcg
aaattccccgtgaccagacttttggacaccaggttggtgaatcagaatgcaagcaggtgggaaagttttgatgtcacccccgctgtgatgcggtgga
ctgcacagggacacgccnaccatggattcgtggtggaagtggcccacttggaggagaaacaaggtgtctccaagagacatgttaggataagcag
gtctttgcaccaagatgaacacagctggtcacagataaggccattgctagtaacttttggccatgatggaaaagggcatcctctccacaaaagagaa
aaacgtcaagccaaacacaaacagcggaaacgccttaagtccagctgtaagagacacccttgtacgtggacttcagtgacgtggggtggaatga
ctggattgtggctccccgggtatcacgccttttactgccacggagaatgccctttcctctggctgatcatctgaactccactaatcatgccattgttc
agacgttggtcaactctgttaactctaagattcctaaggcatgctgtgtcccgacagaactcagtgctatctcgatgctgtaccttgacgagaatgaaa
aggttgtattaaagaactatcaggacatggttgtggagggttgtgggtgtcgctagGATCCggg

IgSP-NS-hBMP-2.seq (SEQ. ID. No. 14)

CCcaagcttGCGTCACCCCTAGAGTCGAGCTGTGACGGTCCTTACAATGAAATGCAGCTGGG
TTATCTTCTTCCTGATGGCAGTGGTTACAGGTAAGGGGCTCCCAAGTCCCAAACTTGAG
GGTCCATAAACTCTGTGACAGTGGCAATCACTTTGCCTTTCTTTCTACAGGGGTGAATTC
Gcaagccaaacacaaacagcggaaacgccttaagtccagctgtaagagacacccttgtacgtggacttcagtgacgtggggtggaatgactgg
attgtggctcccccggggtatcacgccttttactgccacggagaatgccctttcctctggctgatcatctgaactccactaatcatgccattgttcagac
gttggtcaactctgttaactctaagattcctaaggcatgctgtgtcccgacagaactcagtgctatctcgatgctgtaccttgacgagaatgaaaaggtt
gtattaaagaactatcaggacatggttgtggagggttgtgggtgtcgctagGATCCggg

IgSP-KR-hBMP-2.seq (SEQ. ID. No. 15)

CCCaagcttGCGTCACCCCTAGAGTCGAGCTGTGACGGTCCTTACAATGAAATGCAGCTGG
GTTATCTTCTTCCTGATGGCAGTGGTTACAGGTAAGGGGCTCCCAAGTCCCAAACTTGA
GGGTCCATAAACTCTGTGACAGTGGCAATCACTTTGCCTTTCTTTCTACAGGGGTGAATT
CGaaacgtcaagccaaacacaaacagcggaaacgccttaagtccagctgtaagagacacccttgtacgtggacttcagtgacgtggggtggaa
tgactggattgtggctcccccggggtatcacgcctttactgccacggagaatgccctttcctctggctgatcatctgaactccactaatcatgccatt
gttcagacgttggtcaactctgttaactctaagattcctaaggcatgctgtgtcccgacagaactcagtgctatctcgatgctgtaccttgacgagaatg
aaaaggttgtattaaagaactatcaggacatggttgtggagggttgtgggtgtcgctagGATCCggg

IgSP-RRRR-hBMP.seq (SEQ. ID. No. 16)

CCCaagcttGCGTCACCCCTAGAGTCGAGCTGTGACGGTCCTTACAATGAAATGCAGCTGG
GTTATCTTCTTCCTGATGGCAGTGGTTACAGGTAAGGGGCTCCCAAGTCCCAAACTTGA

GGGTCCATAAACTCTGTGACAGTGGCAATCACTTTGCCTTTCTTTCTACAGGGGTGAATT
CGcgccggcgccgacaagccaaacacaaacagcggaaacgccttaagtccagctgtaagagacacccttgtacgtggacttcagtgacgtgg
ggtggaatgactggattgtggctcccccggggtatcacgcctttactgccacggagaatgccctttcctctggctgatcatctgaactccactaatc
atgccattgttcagacgttggtcaactctgttaactctaagattcctaaggcatgctgtgtcccgacagaactcagtgctatctcgatgctgtaccttgac
gagaatgaaaaggttgtattaaagaactatcaggacatggttgtggagggttgtgggtgtcgctagGATCCggg

EXAMPLE 9

Expression of other BMPs

The expression of BMP-2 outlined in Example 8 can also be used to express other BMPs in accordance with this invention, for example by changing the BMP sequence to a sequence that expresses another BMP (such as BMP-2,3,4,5,6,7,8 or 9), or expresses a protein having the activity of a BMP (a morphogen that stimulates the differentiation of an OPC into an osteoblast). Many other transfection protocols are known to those skilled in the art to introduce such sequences into immortalized or conditionally immortalized cells of the osteoblast lineage.

EXAMPLE 10

Expression of Functionally Equivalent BMPs

It will be apparent to one skilled in the art that the bone morphogenetic activity of the BMPs lies not in their precise amino acid sequence, but rather in the epitopes inherent in the amino acid sequences encoded by the DNA sequences. It will therefore also be apparent that it is possible to recreate the bone morphogenetic activity of one of these peptides, without necessarily recreating the exact amino acid sequence. This could be achieved by designing a nucleic acid sequence that encodes for the epitope, but which differs, by reason of the redundancy of the genetic code, from the sequences disclosed herein, while still producing a functional bone morphogenetic protein.

Accordingly, the degeneracy of the genetic code further widens the scope of the present invention as it enables major variations in the nucleotide sequence of a DNA molecule while maintaining the amino acid sequence of the encoded protein. The genetic code and variations in nucleotide codons for particular amino acids is presented in Tables 6 and 7. Based upon the degeneracy of the genetic code, variant DNA molecules may be derived from the DNA sequences disclosed herein using standard DNA mutagenesis techniques, or by synthesis of DNA sequences.

TABLE 6

The Genetic Code

First Position (5' end)	Second Position				Third Position (3' end)
	T	**C**	**A**	**G**	
	Phe	Ser	Tyr	Cys	**T**
	Phe	Ser	Tyr	Cys	**C**
T	Leu	Ser	Stop (och)	Stop	**A**
	Leu	Ser	Stop (amb)	Trp	**G**
	Leu	Pro	His	Arg	**T**
	Leu	Pro	His	Arg	**C**
C	Leu	Pro	Gln	Arg	**A**
	Leu	Pro	Gln	Arg	**G**
	Ile	Thr	Asn	Ser	**T**
	Ile	Thr	Asn	Ser	**C**
A	Ile	Thr	Lys	Arg	**A**
	Met	Thr	Lys	Arg	**G**
	Val	Ala	Asp	Gly	**T**
	Val	Ala	Asp	Gly	**C**
G	Val	Ala	Glu	Gly	**A**
	Val (Met)	Ala	Glu	Gly	**G**

"Stop (och)" stands for the ocre termination triplet, and "Stop (amb)" for the amber. ATG is the most common initiator codon; GTG usually codes for valine, but it can also code for methionine to initiate an mRNA chain.

TABLE 7

The Degeneracy of the Genetic Code

Number of Synonymous Codons	Amino Acid	Total Number of Codons
6	Leu, Ser, Arg	18
4	Gly, Pro, Ala, Val, Thr	20
3	Ile	3
2	Phe, Tyr, Cys, His, Gln, Glu, Asn, Asp, Lys	18
1	Met, Trp	2
Total number of codons for amino acids	61	
Number of codons for termination		3
Total number of codons in genetic code	64	

Additionally, standard mutagenesis techniques may be used to produce peptides which vary in amino acid sequence from the peptides encoded by the DNA molecules disclosed herein. However, such peptides will retain the essential characteristic of the peptides encoded by the DNA molecules disclosed herein, i.e. the ability to induce mesenchymal precursor cells to differentiate into an osteoblastic lineage, and begin the deposition of bone. This characteristic can readily be determined by the assay technique described herein for the detection of characteristic proteins produced by OPCs that have differentiated in this fashion, using the methods of Example 2. Variant peptides include those with variations in amino acid sequence, including minor deletions, additions and substitutions.

While the site for introducing an amino acid sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed protein variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence as described above are well known.

In order to maintain a functional peptide, preferred peptide variants will differ by only a small number of amino acids from the peptides encoded by the native DNA sequences. Preferably, such variants will be amino acid substitutions of single residues. Substitutional variants are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 8 when it is desired to finely modulate the characteristics of the protein. As noted, all such peptide variants are tested to confirm that they retain the ability to induce OPCs to differentiate into an osteoblastic lineage, and initiate bone formation.

The present invention includes OPCs that express any DNA that encodes for a BMP to which the OPC responds by differentiating into a cell demonstrating an osteogenic phenotype.

TABLE 8

Original Residue	Conservative Substitutions
Ala	ser
Arg	lys
Asn	gln, his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu, val
Leu	ile; val
Lys	arg; gln; glu

Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

Changes in biological activity may be made by selecting substitutions that are less conservative than those in Table 8, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. In particular embodiments, at least 90 or 95% of the amino acids in a BMP are identical to the native BMP.

EXAMPLE 11

Dog Derived Osteoblast Precursor Cell (dOPC) that Secretes rhBMP-2

This example describes a conditionally immortalized dog-derived OPC line that can be engineered to secrete rhBMP-2, and can be delivered by the implant to an osseous defect (such as a standardized mandibular defect) in the dog or other animal. It is preferred that the implanted cell be derived from the species into which the implantation occurs, to avoid antibody and complement mediated lysis of the cells. However, a universal donor cell could also be used, regardless of the species. The dOPC line disclosed in this example is derived from a neonatal dog periosteum cell lineage; the derived dOPCs express specific osteoblast-like markers; and dOPCs can be engineered to secrete rhBMP-2 to promote the osteogenic differentiation of the cell, and subsequent accelerated bone formation.

OPCs derived from dog periosteum are isolated by a digestion technique involving a stepwise treatment of approximately 30 minutes each with 0.2% collagenase, followed by 0.25% trypsin. Instead of using four digestions, the cellular preparations from the first and second digestions are plated out. Cells at the time of isolation (P0) are plated at 0.25 x 10^6 in 75 cm^2 tissue culture flasks in alpha MEM with 5% FBS (GIBCO BRL, Inc). The remaining tissue pieces are collected, washed with calcium magnesium free HBSS and digested with 0.25% trypsin-EDTA for 30 minutes. Once the flasks expand to confluence each cell type is subcultured after enzymatic removal with 0.25% trypsin-EDTA to passage 1 (P1). BMP genes can be introudced into the dOPC using the techniques described in Example 5.

Additional characterization and transfection protocols can be performed as in Examples 2-4. The dOPC can be used as an alternative to a human OPC/rhBMP-2 line, such as OPC-1. If the dOPC is used in a human, a short course of immunosuppressive treatment will help assure viability of the recombinant cells in the animal.

Using similar techniques, OPCs derived from monkeys, rats, and a variety of other species can be obtained.

METHODS OF USE

The following Examples 12 to 16 describe how the genetically engineered OPCs of the present invention are used in rats, dogs, and rhesus monkeys, and how they are to be used in humans. In these examples, OPCs that express BMP are placed in critical-sized defects (CSDs) in various bones, such as the mandible and maxilla. Later examples will illustrate the use of a matrix containing OPCs that have not been transformed to express rBMP. However, any of the techniques described in these examples could also be used to introduce the OPCs that have not been transformed.

CSDs are bony defects of a sufficient size that they do not spontaneously heal with new bone formation unless they are treated with a bone promotion formulation, such as the OPCs of the present invention that are engineered to express BMP. The BMP expressing OPCs of the present example (of the matrix localized OPCs of other examples) can be used in a wide variety of anatomical sites, including the flat bones of the skull to treat craniotomy defects, sinus obliteration (for example in the maxillary and frontal sinuses); as therapies for LeFort osteotomy gaps (in the midface); in the mandible, maxilla, and long bones; as a treatment for spine fusions; to improve bone density in osteopenic bones (as in prophylactic treatment of spinal pathological fractures in osteoporosis); and to fill cystic defects.

BMP expressing cells of the osteoblastic lineage (or their precursors such as OPCs) which are responsive to the BMP, are implanted into a bony defect. The cells express a therapeutic amount of the BMP that is sufficient to improve the healing of the defect. The cells can be administered in a variety of delivery systems, including gels suspensions, polymer implants (such as the poly(D,L-lactide) disclosed in Examples 20 and 21), gel components (such as collagen hydrogel cores) of polymer implants as disclosed in Example 20), or a Helistat® collagen sponge, to name but a few examples. The cells can be delivered either by direct surgical implantation into the bone defect, or delivered endoscopically, as described in Example 20. Once implanted, the cells are preferably sufficiently immobilized to be maintained at the site of implantation for about 24-72 hours to initiate the healing process. Immobilization is achieved by a carrier that both localizes and protects the cells. The carrier can also act as a substratum for the attachment of cells, and act as an orientation platform for signaling molecules.

The BMP expressing osteo-committed cells of the invention should produce supraphysiologic amounts of the BMP, sufficient to stimulate osteoblastic differentiation and bone formation in the cells. The local concentration of BMP produced could be, for example, 1-100 mg, more specifically 1-5 mg, or less than about 1 mg, for example less than 500 μg, 35 μg, 1 μg, for example 100 ng or less. A dose of about 0.5 mg rhBMP-2 per ml volume of bony defect is

believed to be appropriate for a young or middle aged healthy adult. A target value for the dose in a typical wound would be, for example, about 5-10 x 10^5 cells/cm^2, or about 1 million cells per ml of bone defect volume. The dose could be increased in patients of increased age, poor health, or in a site that is normally slow to heal. Under these conditions, bone repair would be expected to proceed at a rate of at least about 1μm/day.

EXAMPLE 12

Treatment of Mandibular Defects in Dogs

Dogs are administered antibacterial prophylaxis, intravenous fluids, and anesthesia. After surgical preparation of the surgical site, the tissue overlying the inferior aspect of the left side of the mandible is infiltrated with 3.6 ml of 2% lidocaine hydrochloride with 1:100,000 epinephrine. A 7 cm incision is made along the inferior border of the body of the mandible (a modified Risdon incision), beginning 2 cm from the canine tooth. A full-thickness flap is raised, the mid body of the mandible is visualized, periosteum is dissected from the defect site, and using a high-speed rotary Hall drill with a 703 dental burr and copious sterile water irrigation, a full-thickness bony "saddle defect" with dimensions 2.5 cm in length and 1.0 cm in height of known dimensions is created along the inferior aspect of the mandible. The defect begins 3 cm from the canine tooth and the distal margin is 5.5 cm. After achieving hemostasis, the BMP producing cells are implanted, for example in the implant described in Examples 20 or 21, the soft tissue reapproximated with 3-0 Vicryl, and the incision closed with 2-0 nylon sutures.

EXAMPLE 13

Treatment of Mandibular Defects in Rhesus Monkeys

Rhesus monkeys (weight 6-9 kg) are administered antibacterial prophylaxis, intravenous fluids, and anesthesia as in Example 12 above. A 6 cm incision is made interiorly and medially to the inferior border of the body of the mandible (i.e., a modified Risdon incision) beginning 1 cm from the canine tooth. A full-thickness flap is raised, the mid-body of the mandible visualized, and periosteum dissected from the defect site. Using a high-speed rotary Hall drill with a 703 dental burr as in Example 12, a full-thickness bony saddle defect 1.5 cm long and 1.0 cm high is created along the inferior aspect of the mandible, extending 2-5 cm from the canine tooth. After hemostasis has been achieved, one or more implants are placed in the bony defect as in Example 12, and soft tissue and skin reapproximated.

EXAMPLE 14

Treatment of Maxillary Alveolar Clefts in Dogs

The palatal and mucobuccal fold mucosa circumscribing and contiguous to the right maxillary canine to the left maxillary canine is anesthetized, and full-thickness palatal and

mucobuccal fold flaps are raised to visualize maxillary incisors, the palatine process of the maxilla, and alveolar crestal bone. Two of the three maxillary incisors on each side of the midline are extracted with dental forceps, and a high-speed rotary Hall drill with a 703 dental burr and copious sterile water irrigation is used to prepare a 1.5 cm-wide bony defect extending cephalically from the foramina of the incisive canals to the floor of the nose. A cruciate incision is made and the nasal mucosa is sutured to the oral mucosa with 3-0 Dexon suture to facilitate development of a fistula. A number 6 endotracheal tube (ET-tube) is filled with quick setting, self-curing polymethylmethacrylate to provide a stent that prevents collapse and promotes fistula development.

After three months stents are removed and patency of the fistulas are verified by gentle probing. Several weeks later, the bilateral maxillary cleft defects are incised, granulation tissue removed, and bony walls of the palatine process of the maxillary complex decorticated gently with a high-speed rotary Hall drill with a 703 dental bur and copious sterile water irrigation. Nasal mucosa is sutured with 3-0 Dexon suture to establish a "roof", the implant is inserted, and peridental and palatal mucosa closed with 3-0 Dexon suture.

EXAMPLE 15

Treatment of Maxillary Alveolar Clefts in Rhesus Monkeys

Gingival tissues are gently reflected from the maxillary lateral incisors and canines, and the teeth are extracted with dental forceps. Following extraction, an alveoloplasty is performed using a burr and rotary instruments with copious irrigation (0.9% physiologic saline). Bone is removed unilaterally to the level of the nasal mucosa, and a 1 cm wide oronasal fistula is formed. A number 5 endotracheal tube is inserted through the fistula from the oral opening, into the external nares and through the nasal mucosal defect, then back into the oral opening, to produce a naso-alveolar cleft defect. The tube is filled with poly(methyl methacrylate).

After 8 weeks, the subject is returned to the operating room, the endotracheal tube gently removed, the cleft examined, and the site irrigated with 0.9% physiologic saline. After an additional 4 weeks, the cleft defect is again examined for patency. The implant is then placed into the defect as described in Example 12.

EXAMPLE 16

Treatment of Ostectomy Gaps in Rabbits and Monkeys

The New Zealand white rabbits are anesthetized and the operative site on either the left or right front limb was shaved. A supero-medial incision, approximately 4 cm in length is made and the tissues overlying the diaphysis of the radius dissected away. A 20 millimeter segmental defect is made in the radius with a surgical oscillating saw supplemented by copious 0.9% sterile saline irrigation and the appropriately PDS treatment is placed in the CSD.

Skeletally mature adult male Macaca mulatta (rhesus) monkeys are anesthetized and an incision is made over the radius to expose a full-thickness flap (including periosteum) using gentle, blunt dissection. Radius ostectomies are randomly assigned to either left or right radii, one per animal. The ostectomy is 35 mm long (approximately 4 times the diameter of the radius). Defects are surgically prepared using a nitrogen-driven reciprocating saw with copious irrigation (0.9% sterile physiologic saline). Following removal of the diaphyseal segment, residual bony debris is removed, and the site is stabilized using internal fixation with fracture fixation plates and screws (Leibinger Corporation, Dallas, TX). The appropriate experimental treatment (such as the implant) is placed into each defect, and the wound is closed.

Critical-sized defects (CSDs) are prepared in 60 rabbit skulls, divided evenly among five treatments and two time periods. The rabbits are placed in a supine position, and a full thickness incision down to periosteum is made from the nasal bone to the posterior occipital protuberance in a semilunar design, soft tissues sharp-dissected to visualize the parietal bones, and a 15-mm diameter trephine in a slow speed rotary surgical drill used to prepare a mid-line craniotomy with copious irrigation. Following hemostasis, the implant is inserted into the craniotomy incision.

EXAMPLE 17

Delivery System

When rhBMPs have been used in the past to treat osseous defects, very high (milligram) doses have been required to achieve a therapeutic effect. The present invention takes advantage of a uniquely designed polymer delivery system (PDS) implant that sustains OPCs and localizes rhBMP to enrich local concentrations to promote bone formation. The rhBMP-2 and OPCs, coupled (independently or jointly) to the PDS, constitute a powerful therapeutic package for a broad spectrum of patients.

Two examples of PDS designs are illustrated herein: a cortex-core device (CCD) and an integrated polymer laminate (IPL). The CCD includes a cortex of poly(lactide-co-glycolide) (PLG) containing a known dose of rhBMP-2, which surrounds a gel-like matrix core (for example a hydrogel) with a known quantity of the OPCs of the present invention. The IPL includes alternating PLG and hydrogel laminates with a known rhBMP-2 dose and quantity of OPCs. The PDS strategy fulfills key functions of amplifying a BMP-responsive cell pool and augmenting locally expressed BMP molecules, and is especially relevant for elderly patients who have delayed bone healing, precursor cell decrement, and diminished cell responsiveness. This treatment is therefore especially helpful for the elderly, because the localization of rhBMP-2 and OPCs to enrich local BMP bolsters the BMP-responsive OPC population to enhance the bone regenerative capacity. The PDS also performs a critical function of providing a porous scaffold that supports cell attachment, promotes cell differentiation, orients bone regeneration, and prevents soft tissue

prolapse. The PDS is a substratum that provides a haven for attachment of host pluripotential cells, supports their conversion to osteoblasts, and shuttles the rhBMP and OPCs to the bone defect.

Unlike previous attempts to deliver rhBMP-2 to restore bone and skull defects in rats, sheep and dogs, supra-physiological doses of rhBMP are not needed with the PDS which localizes OPCs and rhBMP, and strategically positions these agents, which decreases dosing requirements. The two specific embodiments of the PDS disclosed in FIGS. 1 and 2 provide a two-component matrix in which the porous PLG scaffold surrounds an inner hydrogel member containing OPCs. The hydrogel carrier supports and localizes the OPCs, while the surrounding PLG (or other polymer) matrix supplies a biodegradable scaffolding that promotes osteoconduction without impairing ultimate bone formation.

Cortex-Core Embodiment

In the embodiment of FIG. 1, a cortex 10 is formed from a mixture of PLG and a BMP, and molded around a hollow core 11. Teflon molds of suitable dimensions are used to cure the polymers in the desired shape, for example at 1 atmosphere of pressure in a vacuum chamber at 15 millitorr at 40°C for 72 hours per each 32 cubic millimeters of cortex volume. The cortex is then loaded with rhBMP-2, for example in a dose of about 100 μg to 500 μg BMP per 1 cubic millimeter of bone volume to be regenerated. The OPCs (or osteoblasts) of the present invention (which express rhBMP) are mixed with a hydrogel in tissue culture 12, and that mixture is then placed in the core 11 of cortex 10 to form an implant 14. Although the quantity of OPCs placed in the hydrogel can vary widely, in this specific example about 100,000 OPCs are provided for each cubic centimeter of bone defect.

The PLG implant, carrying the core of cells, is then placed into an osseous defect, such as the mandibular defect 15 shown in FIG. 1. The cortex will be formed or cut to substantially fill and conform to the edges of the osseous defect. In ablative wounds of long bones, bone fragments may be fixed with fixation plates and screws, and the proximal and distal fragments of bone, once plated, can press fit the implant. Surgical soft tissue closure also secures the implant in place with overlying layers of fascia, tendon, subcuticular tissues and skin. In calvarial wounds (such as trephination defects in flat bone), the implant can be shaped to conform to, and be held in place by, the margins of the wound, and the underlying dura mater and overlying pericranial tissues.

Once the implant is in place, the BMP in the cortex 10 stimulates activity of the OPCs in the core 11, which also produce BMP. The BMP rich environment of the OPCs encourages formation of bone in the surrounding porous PLG matrix, and recruitment of more OPCs from the host. As bone forms, the PLG matrix degrades, which allows bone formation to be completed without interference from the matrix.

Multi-Laminate Implant

FIG. 2 shows an alternative embodiment of the matrix, in which a BMP impregnated sheet 16 of PLG is formed. On top of sheet 16 is then layered a separate layer of hydrogel 17 (such as a collagen gel, agaraose or alginate) in which have been cultured OPCs. Multiple alternating layers of BMP/PLG and OPC/hydrogel form a multi-laminate implant 18. The implant 18 is then placed into a bony defect (such as the mandibular defect 19 shown in FIG. 2), or interposed between the apposed edges of a break in a bone.

The laminate may also be wound into a spiral module 18a in situations where a greater interface surface, a circular cross-section, or more mass transfer capability is desired between the PLG component and the hydrogel layers. The spiral module promotes maximal exchange of signaling components and cell proliferation by communication between the contiguous layers of the spiral. The circular cross-section can also facilitate movement of the implant through a tubular endoscope.

The laminate embodiment of the implant is particularly useful for interpositional bone deficits. The spiral module laminate is also more flexible than the one piece cortex embodiment, hence the laminate can be inserted into deep bony defects having irregular walls, such as clefts in the premaxilla and palate. The spiral module is also useful for placement into irregularly shaped bone defects such as the sinuses of the craniofacial complex, for example sinus defects of the maxilla and frontal sinuses.

The PLG in the implant undergoes non-specific hydrolysis in body fluids. Synthesis and post-synthesis modification of the PDS can also be undertaken to calibrate biodegradation rates of the matrix, so that the matrix degrades at a rate that optimizes the ingrowth of bone into the matrix as it degrades.

The matrix of the polymer (such as the PLG) is sufficiently porous to improve the ingrowth of vascular tissue from host margins, followed by new bone formation (osteoconduction). To help establish optimum pore sizes, disks of poly(D,L-lactide) (DLPL) (3.5 mm-thick) with pore sizes 100 μm, 200 μm, and 350 μm were implanted in rabbits' calvariae. More bone formation was found through disks with 350 μm-sized pores than in the other pores, hence 350 μm pore disks may be used in association with the PDS of the present invention. Bulk erosion of the disks appears to be gradual, and bone ingrowth supports controlled mass loss of DLPL.

The matrix of the present invention can have a variety of chemical compositions, for example a 50:50 poly(D,L-lactide-co-glycolide) (PLG) film or a poly(D,L-lactide) (DLPL) film (e.g., from THM Biomedical, Inc., Duluth, MN). The porous poly(alpha-hydroxy acids) can have a variety of molecular weights, pore sizes, and inherent viscosities, to help fulfill alternative design goals. Representative molecular weights are 10-700 kD, a porosity characterized by a void volume of 70-95%, a pore size range of 15-400 μm, and a pore size distribution in which 75% of the pores

are in the 250-400 μm range and less than about 25% of the pores are within the 15-250 μm range. The inherent viscosity of the porous polymer is, for example, less than about 1.

EXAMPLE 18

Matrix Fabrication

Physicochemical properties of the implant designs promote OPC attachment, support OPC differentiation to osteoblasts, and optimize rhBMP-2 release to promote and support bone formation. To fabricate one such implant, a highly characterized, biodegradable poly(alpha-hydroxy acid) (such as 50:50 PLG) is obtained from a commercial source (e.g, Birmingham Polymers Incorporated, Birmingham, AL) and purified by repeated precipitation in methanol from chloroform solutions. Molecular weights are verified by viscometry at 25°C. Viscosity average molecular weights str calculated from the Marc-Houwink equation: $\lambda = 5.45 \times 10^{-4}M0.73$.

Porous polymer is fabricated by a multiple solvent/thermodynamic procedure in which the poly(alpha-hydroxy acid) (e.g., PLG) is dissolved into methylene chloride/cyclohexane or dioxane/water mixed solvents, and the solutions are frozen at 0 to -4°C, and sublimed under vacuum. The use of these two miscible solvents controls the polymer solution thermodynamics, chain extension and aggregation, and predictably yields a solid-state polymer with suitable pore morphology. Suitably sized Teflon molds are used to cure the polymers and form the sheet or cortex configurations. Pore morphologies can be mapped as a function of solvent compositions and temperature of sublimation. Each of these variables allows modifications in pore engineering, to yield a product with a pore volume of 90% and a size optimized for osteoconduction and osteoinduction.

As a specific example, known quantities of poly(alpha-hydroxy acid)or poly(L-lactide)(PLLA) or poly(D,L-lactide)(PDLLA) can be dissolved in a measured amount of molten naphthalene (at approximately 90°C) and the solution cast into a heated mold and quenched in liquid nitrogen, producing a solid block. The block can be heated at 50°C and 10 millitorr for 12 hours to remove residual naphthalene. Porosity of the product can be controlled by varying the concentration of the polymer in solution, and products with reproducible porosity can be prepared.

A porous matrix can also be made by the techniques described in Mikos et al., *Biomaterials* 14:323 (1993) and *Polymer* 35:1068 (1994). Briefly, the PLLA (or PDLLA) in highly purified form is placed in methylene chloride and vortexed with known concentrations of sodium chloride salt crystals. The concentrations may be, for example, 50, 80 and 90 wt% of the salt:PLLA, and crystal sizes of 300 and 500 μm. The salt suspensions can be cast or spin-coated on to clean glass substrates, and the methylene chloride allowed to evaporate ambiently from covered films. Films can be vacuum-dried to completely remove residual solvent. The films are heated above the PLLA melt temperature (Tm≥180°C) to ensure complete melting of the PLLA-salt crystallites formed on the membrane casing and erase membrane thermal history. The

membranes can then be either quenched in liquid nitrogen, or annealed to room temperature to produce amorphous, semi-crystalline membranes with specific crystalline content.

The PLLA-salt membranes may be immersed in pure deionized water at 25°C for 48 hours to remove the salt, leaving salt-free porous membranes (mesh). The mesh can be air-dried for 24 hours, vacuum dried for 48 hours, and stored in a dessicator under vacuum or dry nitrogen until needed. Membranes made by this method have reproducible properties, with controlled membrane porosity between 300-500 μm for each size of salt crystal, and controlled PLLA crystallinity and degradation characteristics. The porous mesh can be loaded with rhBMP to make the porous matrix of the present invention.

An alternative method for making the porous mesh involves reverse-phase coagulation where a PLLA-salt suspensions is prepared in acetone. This system gels spontaneously at room temperature, however by slowly adding ethanol to the acetone suspension, a porous PLLA mesh membrane can be produced. Acetone and ethanol can be removed under vacuum, yielding a dry, porous PLLA-salt membrane mesh. Distilled water removes the salt, leaving a porous, pliant mesh.

Alternatively, several copolymers of poly(α-hydroxy acid) can be used to prepare the porous matrix. For example, a molar ratio of 1:1 D,L lactide co-glycolide (PLG, described in U.S. Patent No. 4,578,384) with a viscosity of 0.45 dL/g in 1,1,1,3,3,3 hexafluoroisopropanol (HFIP) at 30°C, with a weight average molecular weight of 35 kD can be used. Other copolymers could include different molar rations, such as 3:1, 4:1, and 9:1 PLG. The inherent viscosities may range from 20 kD to 80 kD. In preparing the post-synthesis product, 15 grams of the PLG may be dissolved in 100 mL acetone, stirred for 10 minutes at 25°C, followed by re-precipitation in 100 mL of 100% ethanol. A similar weight to volume ratio may be exploited using methylene chloride followed by re-precipitation with anhydrous methanol. Additional methods useful to the PLG matrix synthesis can be found in Coombes et al., *Biomaterials* 14:297 (1992) and *Biomaterials* 13:217 (1992).

The sample copolymer can be dissolved in dioxane/water or methylene chloride/cyclohexane (96/4 to 95/5, v/v) at a concentration of 10 mg/mL. The solution can be transferred to crystallization dishes and frozen at -24°C and solid disks produced are freeze-dried for 4-5 days. A post-lyophilization vacuum is applied to remove residual solvent, first at room temperature and then at 37°C for two days.

The physical characteristics of the pre-synthesis polymer and post- synthesis product can be derived by using differential scanning calorimetry (DSC). For example, ten mg of well-blended PLLA-naphthalene mixture can be lowered to 90°C and maintained for 5 minutes, followed by slow cooling at 0.2°C/min to 70°C. A phase transition can be noted at the temperature where an exothermic event occurs within this lowering range. The same procedure can be repeated for different PLLA concentrations until the equilibrium versus composition phase

diagram can be constructed. Cooling can be modulated with different combinations of PLLA compositions and time points to examine the physicochemical properties of PLLA after naphthalene sublimation.

Polymers can be characterized to ensure uniformity. Molecular weight can be determined by gel permeation chromatography (GPC) and intrinsic viscosity. GPC can be performed with a Hewlett-Packard 1090M system equipped with column temperature control, a diode array, and a refractive index refractometer. The intrinsic viscosity (iv) measurements can be done in Ubbelohde viscometers in chloroform or dimethyl formamide. The combination of iv measurements and a universal calibration curve based on monodispersed polystyrene can be used to determine the "absolute" molecular weight of the PLLA.

Thermal properties can be evaluated by differential scanning calorimetry (DSC) to derive thermograms and glass transitions (tg) temperatures and crystallinity of the PLLA foams. A Seiko DSC 220 can be used according to ASTM method D3418 to derive the phase diagram for the PLLA-naphthalene mixtures prepared as detailed above. The PLLA products can also be coated with gold in a Hummer C sputter-coater and an AMRay (Series 1810) scanning electron microscope (SEM) can be used to visualize the surface topography, or internal porosity (if freeze fractured). Porosity can be quantitated with a Leica 970 image analyzer, which measures equivalent diameter of pores, pore range, distribution and connectivity. Pore volume and surface areas can be determined by mercury intrusion porosimetry using a Mercury Porosimeter (Model 30K-A-1). Compression measurements can be made following ASTM Method D5024, and compression creep tests by ASTM Method D2990.

EXAMPLE 19

Chemical Modification to Enhance Cell Attachment

The RGD sequence from fibrinogen, fibronectin, and vitronectin are integrin-binding peptide motifs. Massia et al., *Curr. Opin. Cell Biol.* 6:656-662 (1991); Massia et al., *J. Cell Biol.* 114:1089-1093 (1991). Similarly, the peptide fragment p15 (GTPGPQGIAGQRGVV) mimics cell receptor binding activity of type I collagen, and it is this sequence from the type I collagen alpha(I) chain (peptides 766-780) that is associated with cell binding activity of osteoblasts and subsequent osteogenesis. Consequently, the implants of the present invention can include bioactive peptide motifs which are immobilized on the polymer substratum surface to enhance cell accessibility and adhesion. Cell adhesion to the implant's porous matrix can additionally be promoted by copolymerizing lactide monomers with lysine to produce a lysine/lactide random copolymer, as in Cook et al., *J. Biomed. Mater. Res.* 35:513-523 (1997). However, this approach does not preferentially place the cell adhesion molecules at the polymer surface for optimal interaction with OPCs.

RGDs and p15 cell adhesion peptides are produced by standard solid-phase peptide synthesis methods. A fluorinated (preferably perfluorinated) alkyl chain (such as perfluorododecanoic acid) is covalently attached to the peptide amino terminus using standard amide coupling chemistry while the peptide is bead-bound under solid-phase synthesis conditions. The perfluoroalkyl peptide conjugates are cleaved from the solid phase and purified to yield cell adhesion peptides tagged with a chemical marker useful for both polymer surface localization and surface analytical quantification. Schnurer, et al., *Chem. Mater.* 8:1475-1481 (1996); Sun, et al., *J. Am. Chem. Soc.* 118:1856-1866 (1996); Wang, et al., *Supramolec. Sci.* 4:488-497 (1997).

Perfluoro-conjugated RGDs or p15 peptides are dissolved with the selected polymer in methylene chloride solutions and mixed with cyclohexane to produce a two-component solvent-polymer solution for sublimation. Porous foams of these materials are prepared by the dual-solvent sublimation method described in Example 18, using designated peptide loadings.

The internal morphology of the PDS devices can be approached using standard approaches, such as x-ray diffraction, acoustic spectroscopy, and confocal microscopy. Additional analyses may be performed if needed, and these could include dye diffusion, mercury porosimetry, capillary flow porosimetry, and Brunauer-Immett Teller (BET) area measurement to measure continuity of microcellular polymer structure. Other post-synthesis device properties are void size, range, distribution, and interconnectivity.

Using differential scanning calorimetry (DSC), PLG crystallinity is determined. Glass and crystalline transitions and per cent crystallinity are determined from thermal transitions/enthalpic changes in these materials measured on a Perkin-Elmer DSC-7 (heating rate = 10°C/ min). Samples (10-20 mg) are scanned from 20-200°C, cooled and re-scanned to generate endotherms.

Gel permeation chromatography (GPC) can be used to determine homopolymer and copolymer molecular weight distributions using a Hewlett-Packard 1050 HPLC system with a series of Styragel™ size-exclusion GPC columns, refractive index detector, and methylene chloride as the mobile phase. Polystyrene standards are used as calibration references.

Polymer samples are analyzed by SEM after gold-sputtering using a JEOL SEM images are assessed for pore morphology, sizes, distributions, and interconnectivity. Pore size distributions are determined using mercury intrusion porosimetry. Values of pore void fractions and pore area result from such measurements as a function of mercury pressure. Standard analytical methods are adapted to determine porosity, foam density, surface/volume ratio. Helium pycnometry can provide this information.

EXAMPLE 20

Alternative Matrix Designs

Alternative materials for the PLG component (i.e., for both "cortex" and "laminate sheet" embodiments) are commercially available poly(alpha-hydroxy acid) fabrics available from Albany International Corporation, Acton, MA; and Johnson & Johnson, New Brunswick, NJ. Bioresorbable PLG fabrics are produced as velours and weaves that can be post-synthesis-modified to specific textures, thicknesses, fiber diameters, and porosities. Flexible sheets can be cut to size according to the desired shape of the matrix. The fabrics can be surface-modified and coated with cell adhesion peptides to enhance cellular interaction. Surface modification can take the form of (1) coating perfluoroalkylated RGDs or p15 directly onto the fabric surface using organic solvent-based solutions compatible with surface swelling of the PLG fabric, or (2) dissolution of the peptide conjugate, followed by drying to remove the solvent. The conjugates lack appreciable water-solubility and therefore remain hydrophobically tethered to the PLG fabric surface.

When manufacturing some embodiments of the laminates, one goal may be to provide a pliant sheet that is capable of being deformed into a cylindrical shape. To produce such shapes, molecular weight ranges of PLG can be from about 20 kD to 90 kD. However, in some cases, PLG less than about 20 kD may be optimal for biodegradation in osseous wounds of the size described in the Examples. However, these relatively low molecular weights may result in brittle laminate sheets. If the sheets become brittle, they can be reinforced with a porous polyester surgical weave. This strategy will slow biodegradation, but the thin outer scaffolding of the surgical weave provides structural integrity that offsets the slower biodegradation.

The CCD has a PLG cortex containing rhBMP around a core of OPCs in hydrogel. Instead of preforming the core, the cortex may be fabricated as a porous block into which the core is then bored. A known concentration of rhBMP in buffer can be administered to the porous CCD cortex.

EXAMPLE 21

The Hydrogel-OPC Core

The hydrogel carrier for OPCs may be bovine or human type I collagen. Collagen gels or sponges are well-known culture materials and have been shown to regulate cell proliferation, shape and collagen synthesis. Mauch, et al., *Exp. Cell. Res.* 178:493-503 (1988); Nakagawa, et al., *J. Invest. Dermatol.* 93:792-798 (1989); Watt, *TIBS* 11:482-485 (1986). In addition, glycosaminoglycans (GAGs) may be added to the collagen to induce phenotypic differentiation, as in Bouvier et al., *Differentiation* 45:128-137 (1990), and can provide additional properties to enhance osteoneogenesis. Harakas, *Clin. Orthop. Rel. Res.* 188:239-251 (1984).

Type I collagen (Vitrogen®, provided by Collagen Corp.) at 3 mg/ml, is prepared by mixing 8 parts collagen with 1 part 10x PBS, with the pH adjusted to 7.3-7.4 by the addition of 0.1 N NaOH. To achieve a three-dimensional OPC culture within a collagen carrier, OPCs (10^6 cells/ml of gel) are mixed gently into type I collagen solution, set after 45-60 minutes, and are

immersed in alpha MEM containing 5% FBS. Cell culture media is changed 3 times/week to maintain cultures up to 30 days. OPC seeding on top of the type I collagen gels is similar except that the OPCs are plated directly into media on top of the molded collagen gels instead of mixed within the gel.

A known, designated quantity of chondroitin-4 sulfate may be added to selected sets of collagen solutions prior to gel formation and OPC seeding. Glycosaminoglycans (GAGs) such as chondroitin-4 sulfate can be added to enhance progression of OPCs to an osteoblast phenotype.

Many alternative hydrogel materials are candidate carriers for OPCs, such as fibrillar collagen, and polymers such as polyethylene glycol (PEG). However, Type I collagen is preferred because of the ease of adding other components (such as GAGs).

EXAMPLE 22

Preparation of PDS with rhBMP and OPCs

The rhBMP-2 is prepared and incorporated with the polymer under aseptic conditions. Sterilization of materials may be carried out, for example, with ethylene oxide or cobalt-60 gamma irradiation sterilization. Before loading the implants with rhBMP, the implants are sterilized with a bactericidal/viricidal dose of cobalt-60 gamma irradiation. The radiation dose preferably does not exceed 3 Mrad to avoid adverse effects on the properties of the polymer. The product is then stored in a sterile package until needed. The hydrogel compositions are tissue culture quality, and are maintained aseptically. The OPCs are introduced into the hydrogel, and the implant is assembled in the operating room to prevent contamination.

EXAMPLE 23

Pharmacokinetic Determinations

Combinations of selected doses of rhBMP-2 with hOPCs and PLG-hydrogel can be screened to determine an optimum dose of rhBMP needed to promote bone formation in a desired time period. Different doses of rhBMP-2 will cause a dose-dependent expression of osteoblast-like cell markers, which enable pharmacokinetic determinations to be made.

Using a Chinese Hamster ovarian (CHO) cell expression system previously described, rhBMP-2 is produced as a glycosylated 32-kDa homodimer. The amino acid sequence and carbohydrate sites have been determined, as reported in Rosen et al., *Trends in Genetics* 8:97-102 (1992) and Wozney, *Progress in Growth Factors* 1:267-280 (1989). The rhBMP-2 is prepared in a sterile manner, placed in sterile glass vials, closed with rubber stoppers, and freeze-dried.

The rhBMP-2 may be added to the porous polymer either in carboxymethylcellulose (CMC), in a sucrose/arginine (SA) buffer, or in a type I thermal setting collagen (COL). CMC is a water-soluble macromolecule that is prepared as a viscous solution with

known amounts of rhBMP-2, and the solution is then used to impregnate the porous polymer matrix. Sucrose/arginine (SA) buffer solution may also be prepared and used in this manner, particularly because this buffer stabilizes rhBMP.

Under aseptic conditions in a laminar flow hood, known concentrations of rhBMP-2/CMC, rhBMP-2/SA, or rhBMP-2/COL are applied to the polymer "cortex" or laminate "sheets," and pressure is applied to the polymer to ensure homogeneous and complete loading by pore impregnation and polymer adsorption. The impregnated rhBMP-2/PDS component is dried and stored in sterile vials until needed.

Pharmacokinetics of rhBMP-2 release from the PDS is measured with 125I-labeled rhBMP-2 (available from the Genetics Institute, Cambridge, MA., or made using commercially available iodogen reagents) or with an ELISA technique. Following the method of Thies et al., *Endocrinol.* 130:1318-1324 (1992), known concentrations of 125I-rhBMP-2 are added to the porous polymer. Loading of 125I-rhBMP-2 for mid-point pharmacologic activity should be, for example, 100 μg 125I-rhBMP-2/100 mm^2 of each laminate sheet. Three bracketing doses on each side of the estimated pharmacologic dose include: 0, 10, 50, 200, 400, and 800 μg rhBMP-2. Each format of the loaded polymer sheet is placed in appropriate vials containing a 10-fold excess volume of PBS + 1% BSA, and slowly agitated in a circular motion at 37°C. PBS solution is removed for gamma counting and replaced with fresh buffer at the following times: 1,2,4,8,12 hrs; 1,2,4,5,7,14 days. All samples are counted to determine percent cumulative release of 125I-rhBMP-2. Remaining polymer is solubilized to determine mass balance.

Linear regressions and orthogonal contrasts (i.e., Fishers PLSD) are performed to determine the dose of rhBMP-2 producing the optimum in vitro pharmacokinetic profile.

An *in vivo* method can also be used to determine rhBMP-2 dose. PL disks (8 mm in diameter) are loaded with either 0, 10, 50, or 100 μg of rhBMP-2, or rat demineralized bone matrix (DBM) and implanted in the pectoralis major muscles of 54, 35 day-old, Long-Evans rats. At 4 weeks post-implantation, recipient sites are recovered and assayed by previously reported radiomorphometric and histomorphometric methods (Marden et al., *Calcif. Tissue. Int.* 53:262-268 (1993)).

EXAMPLE 24

In Vitro Screening of Implants

CCD and IPL devices may be optimized in a tissue culture setting prior to *in vivo* studies, and multiple configurations can be evaluated for cell proliferation, osteoblast phenotype and ability to mineralize on days 4, 15 and 30. An example of variables that can be tested is shown in TABLE 9.

TABLE 9

In Vitro Assays

Device Configuration	Collagen	GAG	PEG	hBMP-2
CCD1 / IPL1	3 mg/ml	1 mg/ml	(-)	0.5 mg
CCD2 / IPL2	3 mg/ml	1 mg/ml	(-)	1.0 mg
CCD3 / IPL3	2 mg/ml	2 mg/ml	(-)	0.5 mg
CCD4 / IPL4	2 mg/ml	2 mg/ml	(-)	1.0 mg
CCD5 / IPL5	3 mg/ml	1 mg/ml	(+)	0.5 mg
CCD6 / IPL6	3 mg/ml	1 mg/ml	(+)	1.0 mg

The OPC-loaded implant may be evaluated for cell proliferation by the use of a non-destructive colorimetric assay, such as WST-1 (Boehringer Mannheim, Inc.), which determines the number of viable cells that cleave tetrazolium salts added to the tissue culture medium. The amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture system. In addition, one can screen for osteoblast markers such as mineralization, APase expression, and PCR phenotyping for osteocalcin, osteonectin, PTH receptor, and collagen type I (procollagen).

EXAMPLE 25

Use of the Implant in Athymic Rats

The implant of this example includes known doses of rhBMP-2 impregnated into the implant (such as the porous polymer or the gel component), along with the cargo of hOPCs engineered to express BMP-2. This example demonstrates that the implant can deliver hOPCs and rhBMP-2 to a heterotopic wound in the athymic rat, and promote heterotopic ossification (osteoinduction) in a quantity that is dependent on the exogenous BMP dose provided by the implant.

In the operating suite, known doses of aseptically prepared rhBMP-2 are added to pre-measured laminates for the laminate or cortex device. The implant is placed subcutaneously and bilaterally in tissue overlying each pectoralis major muscle. After 14 and 28 days, rats are euthanized, implants recovered, and placed into 70% ethanol for 24 hours. Implants are processed for quantitative radiography and histomorphometry as previously described and the quantity of bone induced by different treatments is determined.

In vivo bone induction is correlated with pharmacokinetic profiles of rhBMP-2/carriers according to the following experiment summarized in TABLE 10.

TABLE 10

Athymic Rat *In Vivo* Bone Information

Group #	Treatment	In Life
1-6	SPS/rhBMP-2 (0,10,50,200,400,800 μg)	2 Weeks
7-12	SPS/OPCs/rhBMP-2 (0,10,50,200,400,800 μg)	2 Weeks
13	positive control*	2 Weeks
14-19	SPS/rhBMP-2 (0,10,50,200,400,800 μg)	4 Weeks
20-25	SPS/OPCs/rhBMP-2 (0,10,50,200,400,800 μg)	4 Weeks
26	positive control*	4 Weeks

8 implants/group

*positive control = rat type I collagen+rhBMP-2

Data from these experiments permits determination of the dose-response curve of various rhBMP-2s on bone induction. However, bone formation varies with anatomical site and species.

Similar trials can be performed in dogs, monkeys, humans and other species, in a variety of sites, to determine optimal doses of OPCs expressing each type of BMP, and optimal amounts (if any) of BMP to be placed in the porous polymer matrix to activate the OPCs in the implant.

Other techniques for assessing bone inductive response include radiomorphometry and histomorphometry.

Radiomorphometry

After 24 hours in 70% ethanol, specimens are radiographed using X-OMAT™ TL high contrast X-ray film (Eastman Kodak Company, Rochester, NY) in a Minishot bench top cabinet (TFI Corporation, West Haven, CT) at 25 KVp, 3 Ma, for 10 seconds. Each roentgenogram is assessed for radiopacity within a standard reference frame superimposed over the specimen. A Leica 970 Image Analysis System (Leica Instruments Inc., Cambridge, England)

measures the area of radiopacity within the standard frame (reported as a percentage of the total area of the frame).

Histomorphometry

After radiographs are obtained, specimens are dehydrated in increasing concentrations of ethanol, followed by embedding in poly(methylmethacrylate), and 4.5-μm coronal sections are prepared and stained with Goldner-Masson trichrome stain (for photomicography and examination of cell and stromal detail) and von Kossa stain. Von Kossa-stained histologic specimens are quantitatively assessed for bone at a standard magnification using a Leica 970 Image Analysis System interfaced with Zeiss Axiophot Microscope (Zeiss Instrument Company, Inc., NY, NY). Using accepted image enhancements techniques (standardized for all specimens), satisfactory optical contrast is achieved for fibrous connective tissue, cartilage, bone, and cellular features by selection of gray level that will be consistent for all sections.

EXAMPLE 26

In vivo Regeneration of Calvarial Critical-Sized Defects in Athymic Rats

This example describes the administration of a specific implant, poly(D,L-lactide), alone or in the presence of rhBMP-2, OPCs, or rhBMP-2 and OPCs, to a calvarial critical-sized defect in athymic rats.

The bone implant was generated from poly(D,L-lactide) (PL) (670 kDa; 0.8 iv) (gift from THM Biomedical, Inc., Duluth, MN, originally supplied by Sofamor Danek, Memphis, TN). All solvents and reagents were ACS grade and purchased from Fisher, Inc., unless otherwise noted. The PL was purified by dissolving in methylene chloride, precipitated in absolute methanol, and dried under vacuum. Following purification, dioxane, which was refluxed and distilled over sodium metal, was utilized to dissolve PL at a concentration 0.01 g/mL (room temperature). The PL solution (80 mL) was poured into a crystallizing dish with a 15 cm diameter and frozen at -20°C. The solvent was removed by freeze-drying under vacuum (<50 mTorr) for 3 days at 0°C. The PL sheets formed were warmed to 23, 30 and 37°C each for 12 hrs to ensure solvent removal.

Discs 8 mm in diameter were prepared from PL sheets, packaged 8-10/container and sterilized with hydrogen peroxide plasma gas (58%) in a Sterrad® 100 Sterilizer using the following cycle: Vacuum Stage, 297 mTorr for 7 minutes; Injection Stage, 7 Torr for 6 minutes; Diffusion Stage, 9.2 Torr for 44 minutes; Plasma Stage, 497 mTorr for 16 minutes; Vent Stage, 4 minutes. The devices were allowed to de-gas for 4 days prior to implantation. Sterility of each run was determined by an American Association of Medical Instrument (AAMI) Biological Standard. The maximum temperature of the run was 43°C.

Purified rhBMP-2 (>98%; lot # 0213J01, provided by Genetics Institute, Andover, MA) was produced as described in Example 23. After reconstituting according to

manufacturer's specifications in a sodium glutamate buffer (5 mM, pH 5.5), appropriate dilutions were placed in sterile glass vials, closed with a rubber stopper, and handled aseptically. To determine the amount of rhBMP-2 to administer, an *in vivo* assay using PL disks (see Example 23), was used. Data analyses favored a 50 μg dose.

Three hours before surgery, the sterilized PL discs were pre-wet with 70% isopropyl alcohol for 15 min and purged 2 times (10 min each) with filter sterilized distilled-deionized water to decrease surface hydrophobicity. The PL discs were washed in HEPES-buffered EBSS, pH 7.4, for 30 min. Two hours prior to surgery, a 22 μL volume of fluid was added to the PL disc to prepare the following 4 treatments: 1) PLC: 11 μL Vitrogen™ (Collagen Corp., Palo Alto, CA), a type I bovine dermal collagen matrix at 3 mg/mL, pH 7.0 in phosphate-buffered saline (PBS) mixed with 11 μL sodium glutamate buffer (5 mM, pH 6.0); 2) PLC/OPC: 11 μL Vitrogen™, 2 x 10^5 OPCs with 11 μL sodium glutamate buffer (5 mM, pH 6.0); 3) PLC/rhBMP-2: 11 μL Vitrogen™, 11 μL of 50 μg rhBMP-2 in sodium glutamate buffer (5 mM, pH 6.0); and 4) PLC/OPCs/rhBMP-2: 11 μL Vitrogen™ and 2 x 10^5 OPCs with 11 μL of 50 μg rhBMP-2 in the sodium glutamate buffer (5 mM, pH 6.0).

The bone biomimetic devices were placed on a petri dish previously treated with Sigmacote™ to inhibit non-specific adsorption of rhBMP-2, followed by placement in a humidified 37°C incubator for 90 min prior to implantation to allow thermal gelation of the Vitrogen™. Bone implant devices were transported aseptically to the surgical suite for implantation.

Following aseptic procedures, an 8 mm diameter defect was prepared in the calvarial bone with an 8 mm trephine and copious irrigation with physiologic saline. The craniotomy segment with attached periosteum was removed gently, leaving the dura intact and 1 of the 4 designated treatments was inserted. The PLC implants were retained at the site by surrounding soft tissues, which were closed with 4-0 sutures. Rats were euthanized at 2 and 4 weeks post-operatively, calvarectomies were accomplished, and tissues were prepared and assessed for radiomorphometry and histomorphometry as described in Example 25. Data were analyzed by multiple analysis of variance (ANOVA) and Fisher's protected least significant difference test for multiple comparisons to determine differences among treatments and between time periods. Statistical significance was established at $p \leq 0.05$.

The fabricated PL exhibited an interconnecting open-pore meshwork, which allows cellular access for penetration, growth, and differentiation. The 8 mm diameter PL device had an average unit mass of 21.7 mg and a void volume approximating 85-90%. Quantitative scanning electron microscopic (SEM) assessment of PL scaffolds revealed a porous structure with pores between 50-250 μm. Although plasma gas sterilization resulted in 20-25% shrinkage, after wetting and rehydration the scaffolds regained their pre-sterilization architecture and morphology.

The experimental bone implants were convenient to manage at surgery and were inserted easily into the craniotomy defects, retained within the bone margins, provided hemostasis

control, and prevented soft tissue prolapse. No mortality occurred throughout the course of the study and tissue healing was unremarkable.

By week 2, PLC/OPC, PLC/rhBMP-2 and PLC/OPC/rhBMP-2 treatment groups displayed more radiopacity compared to the PLC alone. This result was confirmed with radiomorphometric data (FIG. 8). The PLC/OPCs exhibited a mean radiopacity of 38% versus 22% for PLC. Radiopacity superior to PLC alone was also observed for PLC/rhBMP-2 and PLC/OPC/rhBMP-2. A histological analysis at 2 weeks revealed some new bone formation in the defects treated with either PLC or PLC/OPCs, and fibrotic tissue prevailed. Numerous multinucleated giant cells were observed in PLC/OPC-treated sites. Remnants of PLC could not be detected either with brightfield or polarization microscopy techniques. Defects implanted with PLC/BMP-2 and PLC/BMP/OPC had numerous bony trabeculae, a greater amount of new bone, as well as a consolidation of lamellar bone along the dural aspect. Histomorphometric data for new bone formation corroborated the histological observations (FIG. 9).

By week 4, PLC/OPC, PLC/rhBMP-2 and PLC/OPC/rhBMP-2 treatments had more radiopacity than PLC. The PLC/rhBMP-2 had a significantly greater percent radiopacity than other groups (FIG. 8). In addition, PLC/OPCs/rhBMP-2 had significantly more radiopacity than either the PLC or PLC/OPCs. Moreover, defects treated with PLC/rhBMP-2 had a time-dependent increase in percent radiopacity from 2 to 4 weeks. At 4 weeks, the histological profile among treatments was similar to the 2 week profile. However, the quantity of new bone for the PLC/BMP and PLC/BMP/OPC groups was greater than at 2 weeks (FIG. 9), and the appearance of the new bone in the PLC/BMP/OPC at 4 weeks was more lamellar than for the same treatment at 2 weeks. While multinucleated giant cells were evident at 4 weeks in PLC/OPC-treated defects, this cell phenotype was absent in PLC/BMP and PLC/BMP/OPC sites at 4 weeks. These results demonstrate that the tissue-engineered bone implants promote time-dependent bone regeneration in calvarial CSDs in athymic rats.

Although PLC/OPCs at 4 weeks did not inspire as robust a response, boosting cell-loading from 10^5 to 10^6 (a one log increase) is expected to provide more bone formation. In addition, the PLC/OPCs/rhBMP-2 response can be increased by a log increase in OPC quantity. A seeding density of 2×10^5 OPCs per bone implant was used, which is considerably less than the quantity of cells implanted previously: 5×10^6 for W-20-BMP-2 producing cells implanted into an 8 mm femoral gap model in athymic rats (Lieberman et al., *J. Orthop. Res.*, 16:330-339 (1998)), 7.5×10^6 for human MSCs delivered with a ceramic carrier into athymic rats, and 7.5×10^6 dog MSCs applied with a ceramic to a femoral dog gap model (Bruder et al. *J Bone joint Surg.* 80A:985-996 (1998)). Consequently, a log increase in OPC cell density may enhance the effect of both PL/OPC and PL/OPC/rhBMP-2 on bone regeneration.

In conclusion, this example demonstrates for the first time that a combination of PLC/OPC or PLC/OPCs/rhBMP-2 can regenerate bone in calvarial CSDs. While untreated CSDs

were not part of the experimental design, substantial, well-documented previous data clearly and unambiguously confirm untreated 8 mm-diameter CSDs in rats will not heal with new bone formation.

EXAMPLE 27

Endoscopic Delivery of OPCs

This example discloses an improved method and endoscope device for delivering the OPCs into bone to heal bony defects or promote the growth of denser bones in osteopenic areas. An overview of the method is disclosed in FIGS. 4-6, which illustrate the introduction of OPCs through a cannula 42, endoscope 46, and/or balloon catheter 48, into an area of osteoporotic vertebral trabeculae 24. FIGS. 10A-10D show specific embodiments of such devices.

Delivery of OPCs

FIG. 4A shows a spinal vertebrae 20 having a cortex 22 surrounding a less dense cancellous bone (trabecular) portion 24. Each vertebra also includes a pedicle 26, a transverse process 28, a spinous process 30, and a lamina 31 extending between the pedicle 26 and spinous process 30. These structures form a vertebral canal, surrounding and enclosing the spinal cord 32 in a protective casing to avoid injury to the delicate neural tissue of the spinal cord. FIG. 4A illustrates an osteoporotic vertebral body 20 in which the cancellous bone 24 in the body 20 has become less dense (as shown in the photomicrographic enlargement of a portion 36 of the cancellous bone of the vertebral body 24). This loss of bone density predisposes the body 20 to pathological fractures which are painful, contribute to compression of the spinal column, and can lead to spinal cord injury with consequent neurological impairment.

To deliver the OPCs of the present invention into the cancellous bone of the vertebral body 24, a rigid, sharp-tipped instrument (such as K wire 40 in FIG. 4A) is introduced percutaneously through the skin and into the bone of the pedicle 26, or lateral vertebral wall 22. After the K wire 40 is placed, the tract is dilated with dilators up to the diameter of a rigid cannula 42. The cannula 42 is then inserted over the K wire 40 and the K wire 40 is removed leaving the cannula 42 in place to provide a passageway to the body 20. An endoscopic drill 44 is introduced through cannula 42 to drill a pathway through the pedicle 26 into the vertebral body 20 (FIG. 4B). If a lateral approach is chosen, the drill will penetrate the lateral cortical wall of the vertebral body 22.

The cannula 42 is advanced along with the endoscopic drill 44 through the pedicle 26 or lateral vertebral wall 22 until the cancellous bone of the vertebral body 24 is encountered. The endoscopic drill 44 is a specially configured drill where there is a small fiberoptic endoscope in the center of the drill bit. This endoscopic drill 44 allows the user to view the path of entry of the drill, so that the drill 44 can be advanced through the pedicle 26 or vertebral wall 22 in a highly

controlled fashion. This limits the potential for entering the vertebral foramen or spinal canal, which can lead to damage of the spinal cord 32 or nerve root.

FIG. 4C demonstrates the introduction of a steerable endoscope 46, through a cannula 42 into the vertebral body 20. A balloon catheter 48 can be introduced through the steerable endoscope 46 into the cancellous bone of the vertebral body 24. The route of the catheter 48 will be guided by the steerable endoscope 46. The balloon catheter 48 has one or more side vents 50 (FIGS. 5 and 6) through which OPCs can be delivered into the cancellous bone of the vertebral body 24. The vents 50 have dimensions of a sufficient size (e.g., 20 μm i.d.) to provide free passage of the cells from the endoscope/catheter unit without physically disrupting the cells. Once the balloon catheter 48 has been endoscopically guided to the proper predetermined location in the vertebral body 20, the OPCs can be delivered through the balloon catheter 48 into the cancellous bone 24. The cells may be introduced while suspended in a hydrogel, or in a calcium-phosphate based commercially available "ceramic" preparation (such as Bone Source™ HA cement, from LEIBINGER, Dallas, TX, or Alpha BSM™ from ETEX Corporation of Cambridge, MA.) which may be gently moved through the steerable endoscope 46 by an auger mechanism (FIG. 10B) that extends through the balloon catheter 48. Alternatively, the OPCs can be placed in the protective cortex core or multi-lamellar matrix embodiment (wound into a spiral with a circular cross section) and introduced under pressure through the balloon catheter 48. The matrix embodiment provides additional protection to the OPCs during their passage through the balloon catheter 48, and introduction into bone.

Areas in which OPCs have already been deposited are illustrated in black dots in FIG. 4C, and the photomicrographic enlargement of that area shows that the trabeculae 24 provide a porous structure in which the OPCs (or the implant matrix) is supported. The balloon catheter 48 can be advanced and retracted to deposit the OPCs at multiple spaced or predetermined locations throughout the vertebral body 20. If desired, the cancellous (trabecular) bone 24 in the vicinity of the catheter tip can be gently compressed by inflating the balloon catheter 48, or by windshield wiper-like motion of the steerable endoscope 46. This local trabecular compression creates a small cavity into which the OPCs can be introduced without creating excessive back-pressure in the balloon catheter 48 that requires forcing the cells out of the balloon catheter 48 into the bony matrix under a large positive pressure.

The steerable endoscope 46 has a flexible tip that can be oriented by manipulation of external controls to determine the direction the endoscope points. As shown in FIG. 6, the balloon catheter 48 can then be advanced through the steerable endoscope 46 until it reaches a position where the OPCs are to be deposited. At this point, the tip of the balloon catheter 48 is inflated to gently compress surrounding bone. The balloon is deflated, leaving a small cavity into which the OPCs are introduced through vent openings 50. The balloon catheter 48 is then

withdrawn through the steerable endoscope 46 and then the endoscope is withdrawn through cannula 42.

EXAMPLE 28

Specific Delivery Embodiments

A particular embodiment of a coxaxial cannula 100 for delivering OPCs into bone is illustrated in FIG. 10A. Once an endoscopic drill has provided an access port into bone of the vertebral body 20, the coxaxial cannula 100 is introduced through cannula 42 into the vertebral body 20 (FIG. 4B). This coaxial cannula 100 can be made of any suitable sterilizable material (such as plastic or stainless steel). The cannula 100 has a cylindrical inner wall 101 that defines an inner (central) lumen 102 and a cylindrical outer wall 103 that is coaxial with the inner wall 101 such that an outer (peripheral) lumen 104 is defined between the inner and outer walls. The outer lumen is divided into sections 106, 108, 110 and 112 by continuous partitions 114, 116, 118 and 120 that extend longitudinally or helically along substantially the length of cannula 100. The illustrated cannula 100 has an outer lumen that is divided into four sections. There can be as few as two sections and potentially an infinite number. The inner lumen and the sections of the outer lumen serve as longitudinal passageways through the cannula 100.

A steerable endoscope 122 (FIG. 10C), having a steerable tip 124 (FIG. 10D), can be introduced through the inner lumen 102 of cannula 100 (FIG. 10A). In particular embodiments, OPCs can be introduced through the lumen of the endoscope 122. The cannula sections between the inner and outer walls (FIG. 10A) allow pressurized air and bone barrow/blood to escape, preventing barotrauma to the OPCs that are introduced into the vertebral body 20 (FIG. 4) and preventing venous embolism.

Another embodiment of a delivery system is shown in FIGS. 10B and 10D, in which the steerable endoscope 122 is provided with an inner cartridge unit 126. This cartridge unit is composed of a reservoir 134 connected with a flexible tubular outer body or cannula 128 containing a helical screw/auger 130. The auger 130 is of sufficient diameter in relation to the inside diameter of the cannula 128 that rotation of the auger can transport liquid or particulate material through the cannula. As illustrated in FIG. 10D, the coaxial cannula 100 contains the steerable endoscope 122, which in turn contains the cannula 128 of the cartridge unit 126. By using these components together, excessive pressure in the bone (which can damage OPCs or cause venous embolization) can be avoided because the pressure will be vented through the outer lumen 104 of cannula 100.

The cartridge unit 126 is loaded with OPCs. By either manual or motorized rotation of the auger screw 130 (FIG. 10 B), the OPCs are gently advanced into the vertebral body 24 (FIG. 4). The helical screw 130 may be made as a twisted-in-wire or other form of screw or brush. The material making up the auger must be sterilizable and not harm the OPCs (could be

stainless steel, plastic or other material). The auger will be flexible so that it can bend within the steerable endoscope 122. In order to keep the auger from collapsing within the cannula 128 when it is bent within the steerable endoscope 122, a portion of the screwblade or brush bristle will be made of a stiffer material so as to maintain the augers location in the middle of the cannula 128.

Alternatively, the cartridge unit 126 could be used to deliver a great range of biologic compounds, plastics or other materials that might be administered into the cancellous component of any bone in the body. In addition, the cartridge unit 126 could be used to deliver materials to any bone in the body directly, without an endoscope. For example, the cartridge unit 128 could be used to deliver bone marrow.

EXAMPLE 29

BMP Expression Systems

A variety of expression systems which can be used for production of recombinant BMPs is shown in Table 11.

TABLE 11

Vector*	Expression system	Product	Reference
pMT2CX plasmid	monkey COS-1 cells	rhBMP-1, 2 and 3	Wozney et al 1983
pMT2CX plasmid	CHO1 cells E.coli	rhBMP-1, 2 and 3	Wozney et al. 1988
Xenopus BMP-4 cDNA under control of CMV-promoter	COS-1 cells	rxBMP-4	Koster et al. 1991
	CHO cells	BMP-2	Israel et al. 1992
pMBC-2T-f1	murine mesenchymal progenitor cell line C3Hl0T1 /2	rhBMP-2 and 4	Ahrens et al 1993
pCVD(X) + SV40 promoter + CMV-promoter + dbfr-gene pSVD(X)+ SV40 promoter + dhfr-gene	CHOcells	rxBMP-4	Suzuki et al. 1993
pUC19 and pdKCR-dehydrofolate reductase (dhfr)	CHO cells	murine rBMP-4	Takaoka et al. 1993
pAG60 with neomycin resistance gene, cotransfection with DVR-6 gene, under control of keratin III and IV regulation element	BMGE+H cell line	human rDVR-6	Wall et al. 1993
	C3Hl0T1/2 mesenchymal cell line	rhBMP-2	Wang et al. 1993
Co-transfection with BmNPV (strain PGE) and pBm4-hBMP2	Silworm larvae	rhBMP-2	Ishida et al. 1994
pDSRα	CHO(DG44) cells	BMP-2 and BMP-4	Koenig et al 1994
pJT4	COS-7	BRK-1 and DAF-4 receptor proteins	
PMS32C/BMP1 plasmid	E. coli	human BMP	Ma et al 1994

EMC G5	CHO	rhBMP-2	Marden 1994
pUC19 and pdKCR-dehydrofolate reductase (dhfr)	CHO cells expanded and inoculated into the hindlimbs of nude mice	murine rBMP-4	Shimitzu et al. 1994
pUC19 and pdKCR-dehydrofolate reductase (dhfr)	CHO cells without and in diffusion chambers s.c. in nude mice	murine rBMP-4	Takaoka et al. 1994
pVL1393 & AcNPVco-tranfection	Insect Sf9 cells	rxBMP-2 rxBMP-4 rxBMP-7	Hazama et. al 1995
pBlueBacII & AcNPV co-transfection	Insect Sf9 cells	rhBMP-2	Maruoka et al 1995
vector = ??	E. coli	rhBMP-2	Ruppert et al. 1996
pRK7	CHO cells	murine vgr-1 (BMP-6)	Gitelman et al 1994
αGlyTag plasmid	Transgenic mice	BMP-2-T-Ag fusion protein	Ghosh-Choudhury et al. 1996

*Nomenclature does not follow uniform criteria; either the original names of the plasmids or the names after ligation of BMP-cDNA have been used.

In view of the many possible embodiments to which the principles of our invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as a limitation on the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A composition for treating osseous defects, comprising a porous matrix and a therapeutically effective amount of a cell that is committed to an osteogenic lineage.

2. The composition of claim 1, wherein the cell that is committed to an osteogenic lineage is a conditionally immortalized osteoblast precursor cell having the identifying characteristics of osteogenic precursor cell line 1 (OPC1).

3. The composition of claim 1, further comprising a therapeutically effective amount of a bone morphogenetic protein (BMP) which induces bone formation by the osteogenic precursor cell.

4. The composition of claim 3, wherein the BMP is BMP-2.

5. The composition of claim 4, wherein the BMP-2 is recombinant BMP-2 expressed in a therapeutically effective amount by OPC1.

6. The composition of claim 1, wherein the porous matrix comprises poly (D,L-lactide) and collagen.

7. A method of treating an osseous defect by introducing into the defect the composition of claim 1.

8. A method of treating an osseous defect by introducing into the defect the composition of claim 2.

9. A method of treating an osseous defect by introducing into the defect the composition of claim 3.

10. A method of treating an osseous defect by introducing into the defect the composition of claim 4.

11. A method of treating an osseous defect by introducing into the defect the composition of claim 5.

12. A method of treating an osseous defect by introducing into the defect the composition of claim 6.

13. A method of treating an osseous defect by introducing into the defect a cell that has committed to an osteogenic lineage, and expresses an osteogenic bone morphogenetic protein which induces bone formation by the cell.

14. The method of claim 13, wherein the bone morphogenetic protein is BMP-2.

15. The method of claim 13, wherein the cell is an osteoprogenitor cell that is conditionally immortalized.

16. A conditionally immortalized osteoblast precursor cell having the identifying characteristics of OPC1.

17. An implant for administering osteogenic precursor cells to a region of a bony defect, comprising a cushioning component protected by a porous osteoconductive biodegradable protective component.

18. The implant of claim 17, wherein the cushioning component is a hydrogel, and the porous protective osteoconductive component is a poly(α-hydroxy acid) matrix.

19. The implant of claim 17, wherein the protective component is impregnated with an osteogenic bone morphogenetic protein.

20. The implant of claim 17, wherein the osteogenic precursor cell has been transfected with a vector to express therapeutically effective amounts of a bone morphogenetic protein sufficient to promote osteogenic differentiation and bone formation by the osteogenic precursor cell.

21. An instrument for treating a bone, comprising:

a supply of osteogenic precursor cells; and

a cannula for delivery of the osteogenic precursor cells into the bone.

22. An instrument comprising a catheter that is sized and shaped to deliver the implant of claim 17.

23. The instrument of claim 21, comprising an endoscope.

24. A method of treating bone deficits comprising delivering into bone a material that promotes bone formation.

25. The method of claim 24, wherein the material is an immortalized osteoblast precursor cell into which has been introduced a vector for the expression of a bone morphogenetic protein.

26. The method of claim 24, wherein the material is an implant that contains an exogenous, therapeutically effective dose of osteoblast precursor cells and a bone morphogenetic protein, effective to promote osteogenesis.

27. The method of claim 24, wherein the bone deficit is caused by osteoporosis, cyst-like cavities, surgical resection, traumatic avulsion, and congenital insufficiency.

28. A cannula comprising a central lumen and a peripheral lumen.

29. The cannula of claim 28, wherein the peripheral lumen comprises plural lumena.

30. A cartridge unit comprising:

a cannula; and

a rotatable member having a peripheral helical screw, the rotatable member extending through a cannula.

31. The cartridge unit of claim 30, further comprising a driver for rotating the rotatable member.

32. The cartridge unit of claim 30, further comprising a reservoir attached to one end of the cannula for supplying osteoblast precursor cells to the helical screw.

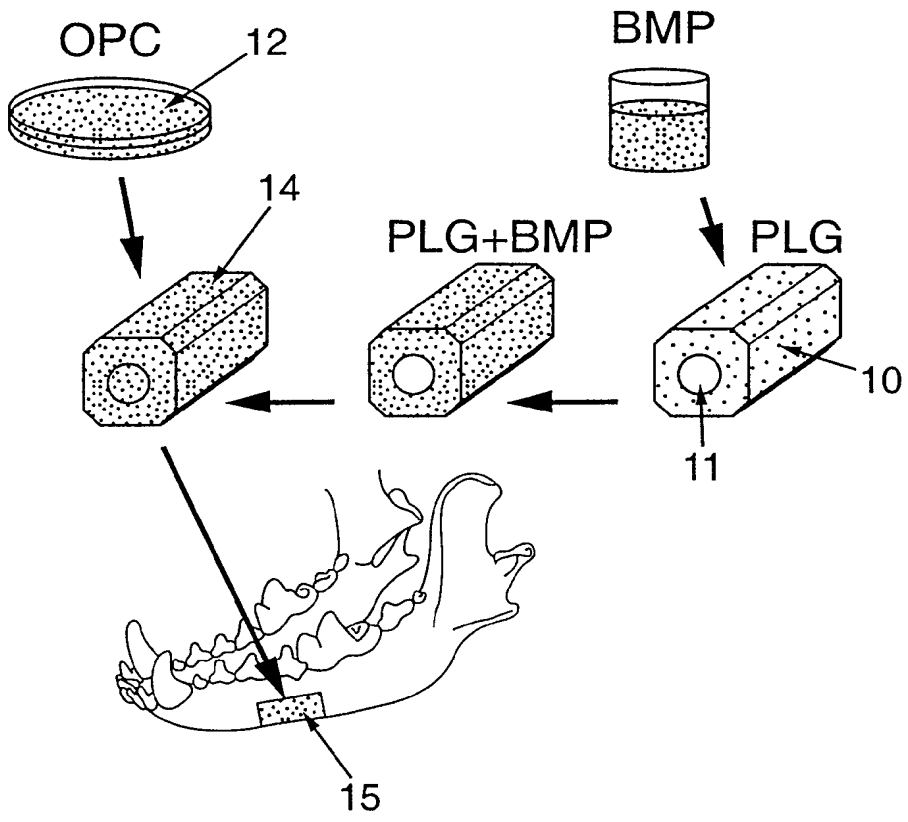
33. A method for delivering a flowable substance into a region inside a body of a living subject, the method comprising:

inserting an outer cannula into the body so that the cannula extends from a location outside the body to a target location inside the body;

providing an inner cannula inside the outer cannula, the inner cannula defining plural longitudinal passageways;

passing a flowable treatment substance into the body through one of the passageways while allowing material to flow out of the body through another of the passageways.

FIG. 1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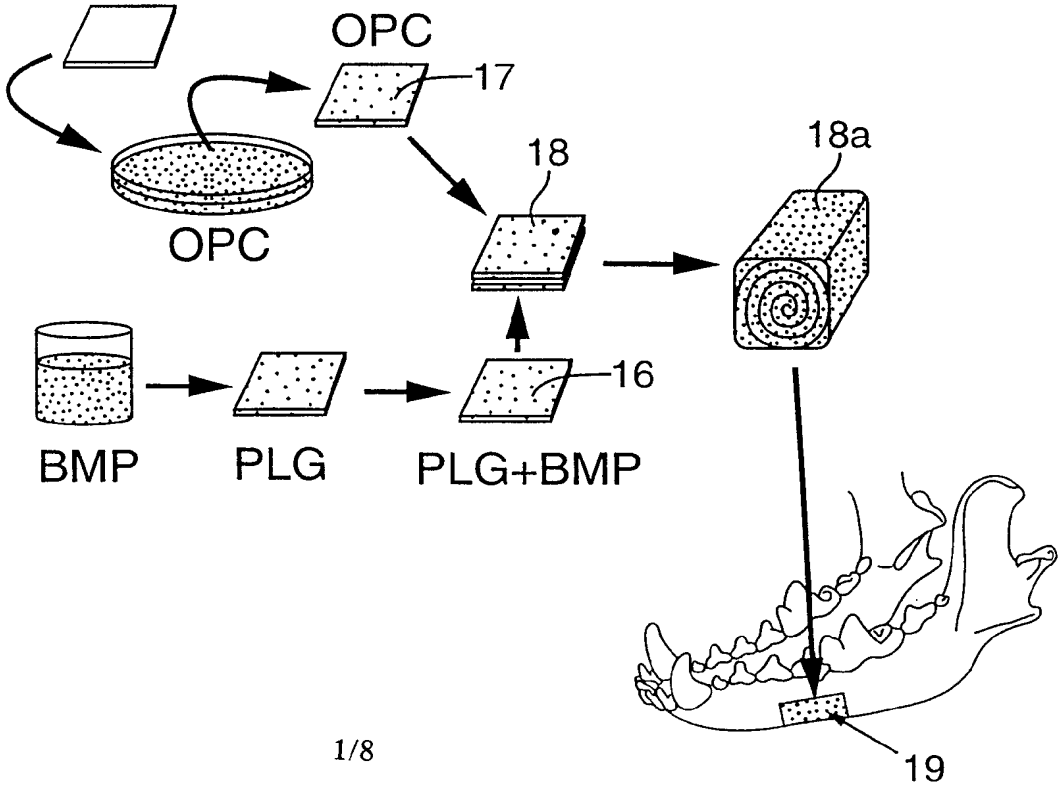
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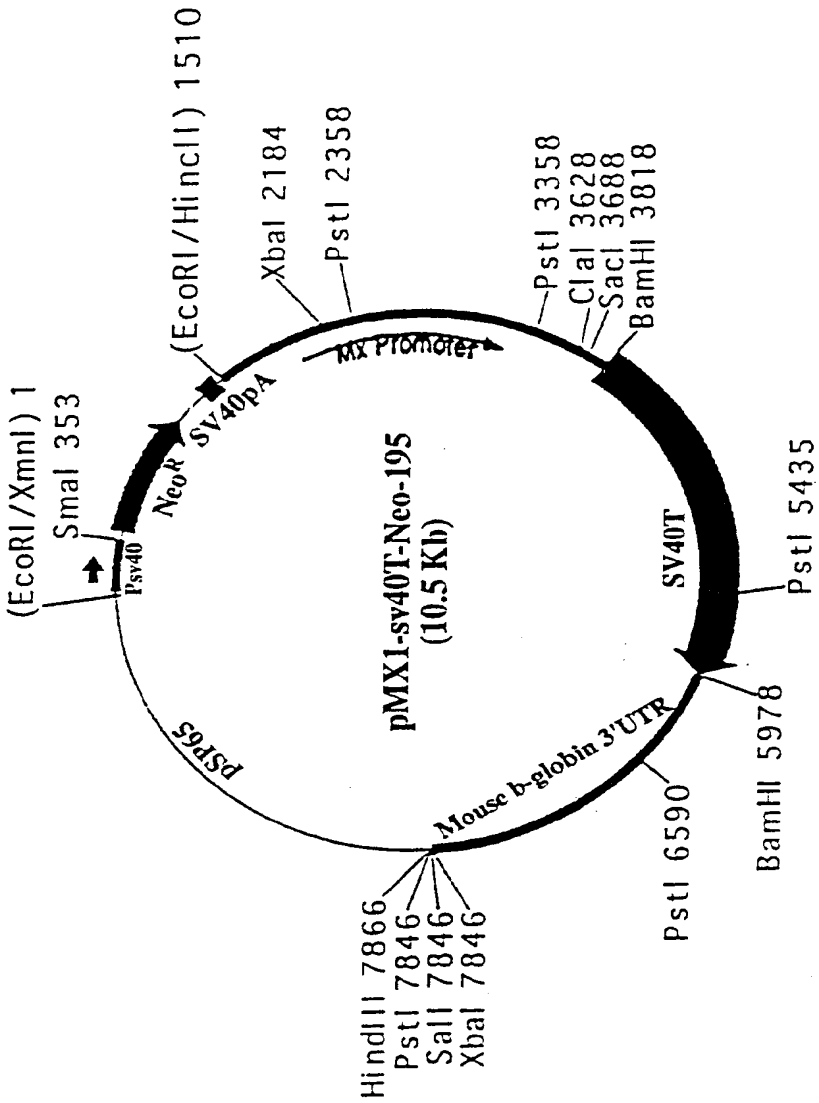
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HindIII 7866
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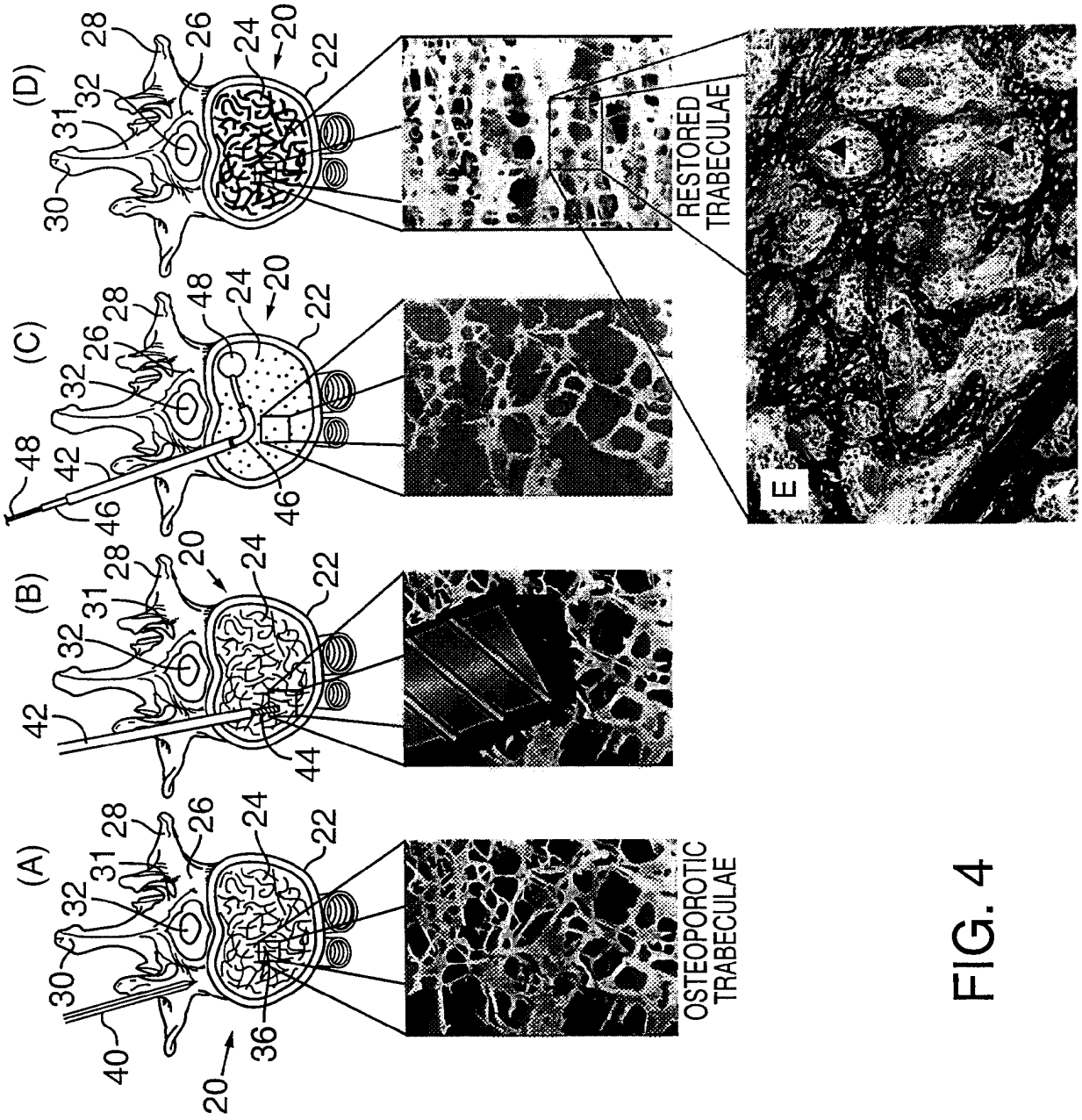
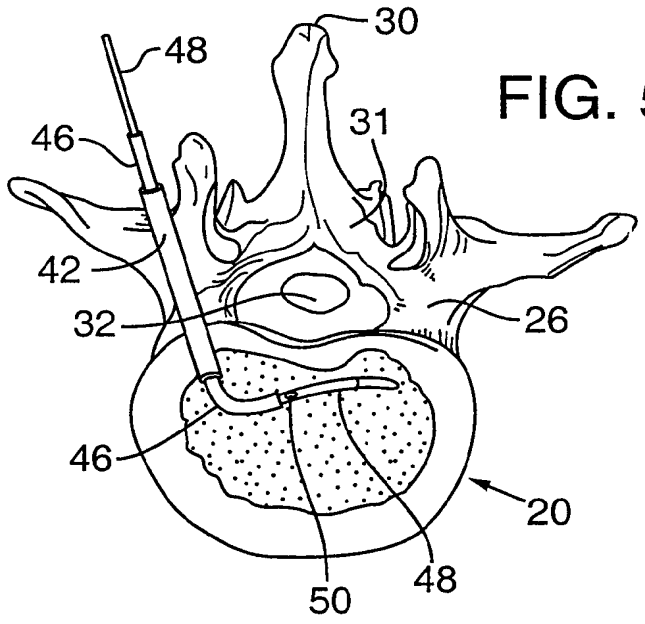


FIG. 4



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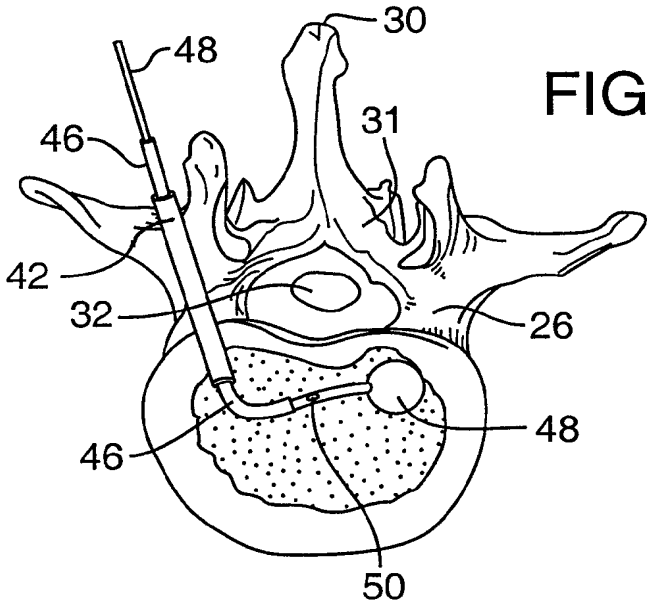
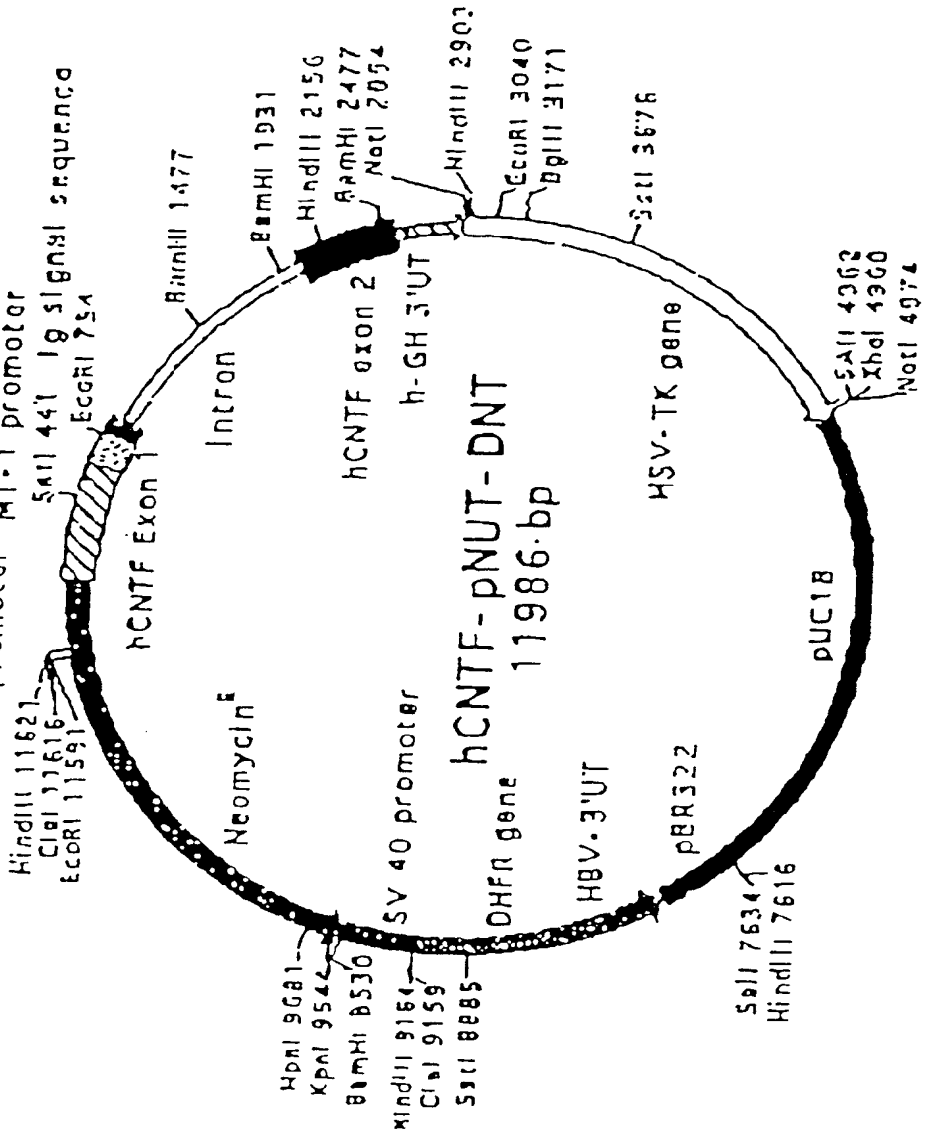


FIG. 6

FIG. 7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BamHI 1931
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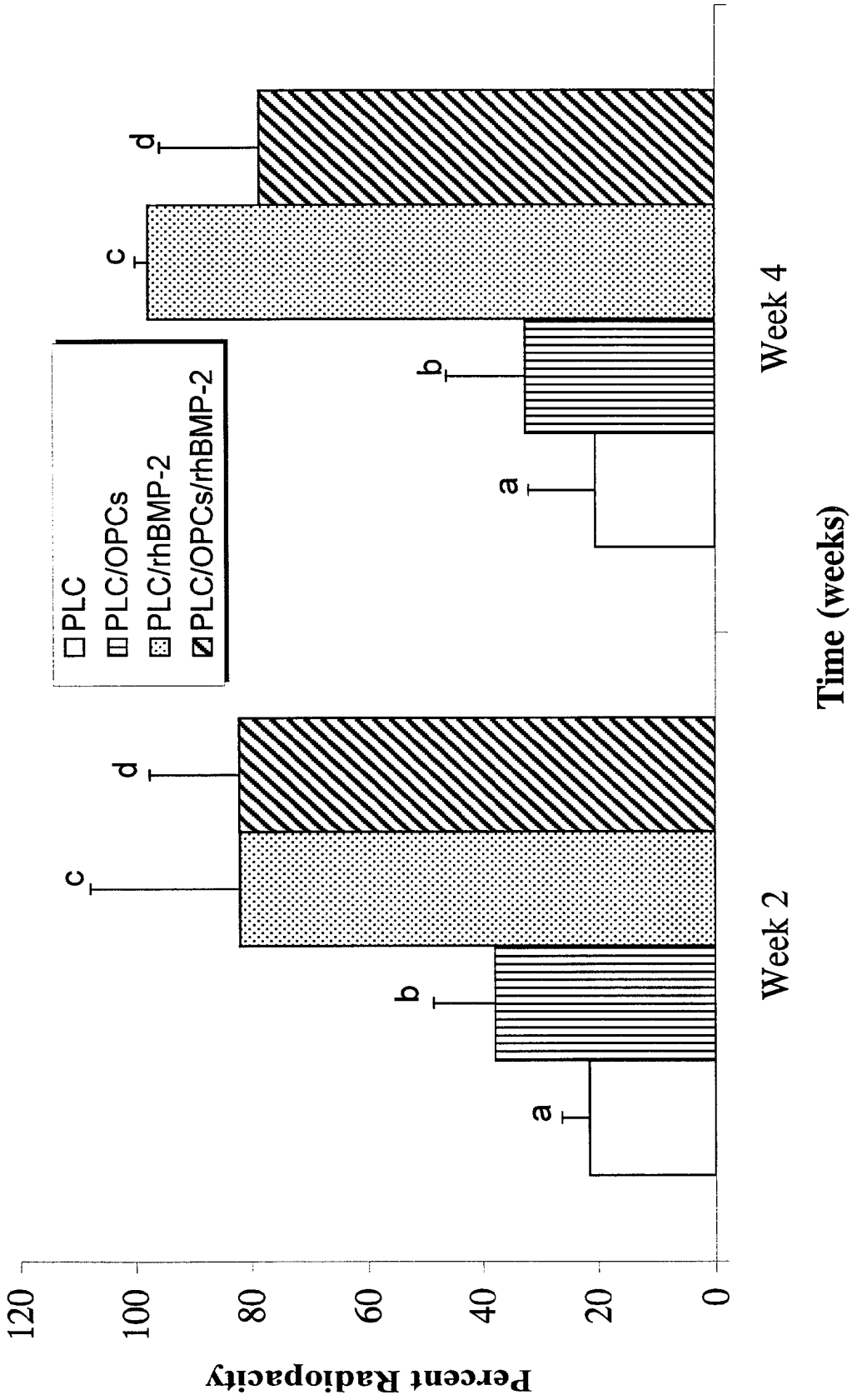
FIG. 8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20
40
60
80
100
120
Week 2
Week 4
Time (weeks)
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b
c
d
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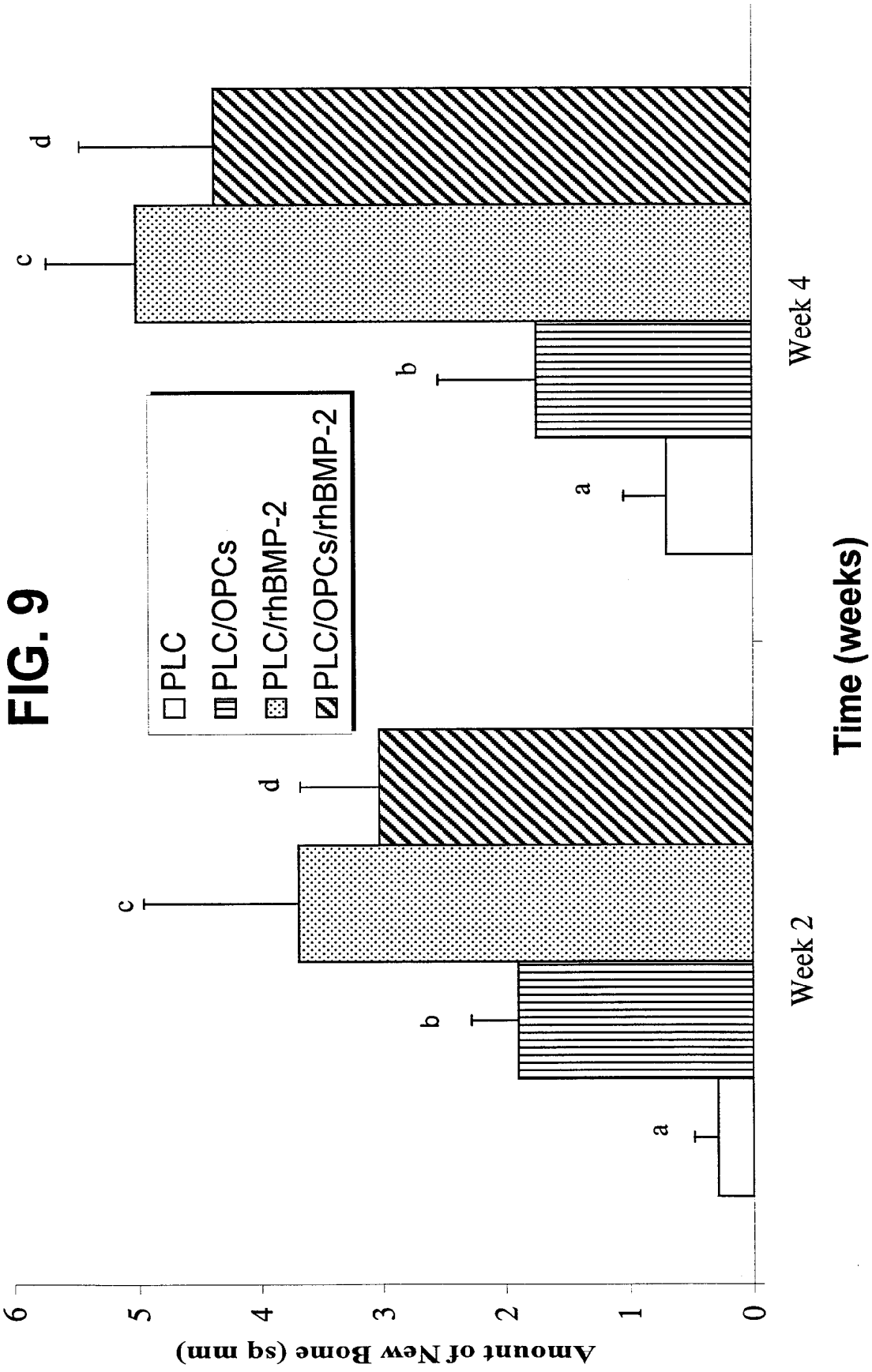
PLC/rhBMP-2
PLC/OPCs/rhBMP-2

FIG. 9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0
1
2
3
4
5
6
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PLC/rhBMP-2
PLC/OPCs/rhBMP-2
a
b
c
d
Week 2
Week 4
Time (weeks)

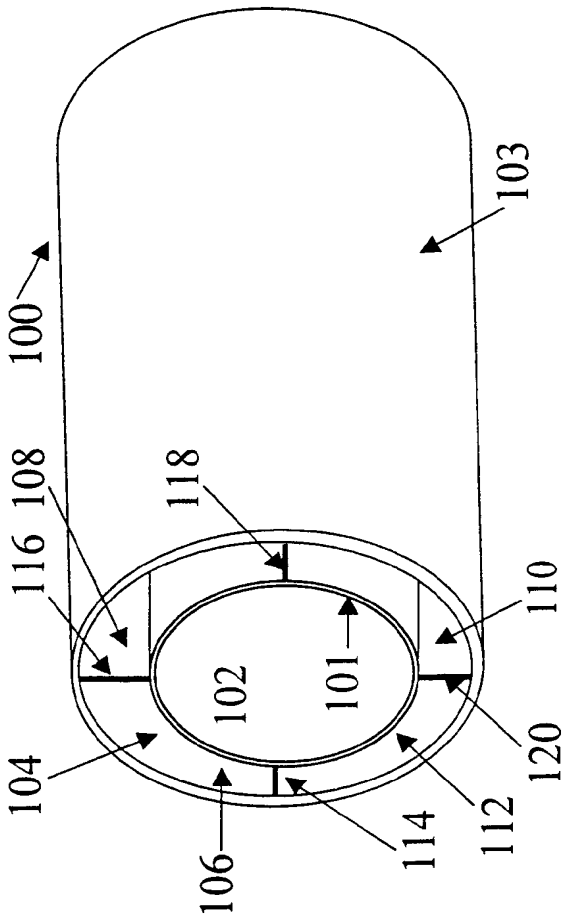

FIG. 10A

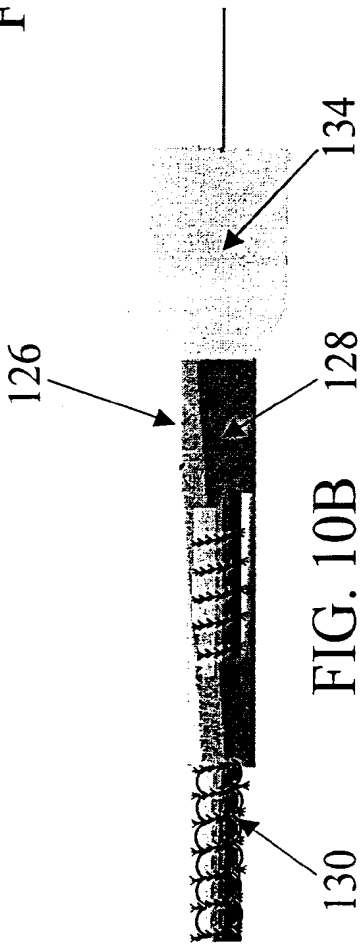

FIG. 10B

FIG. 10C

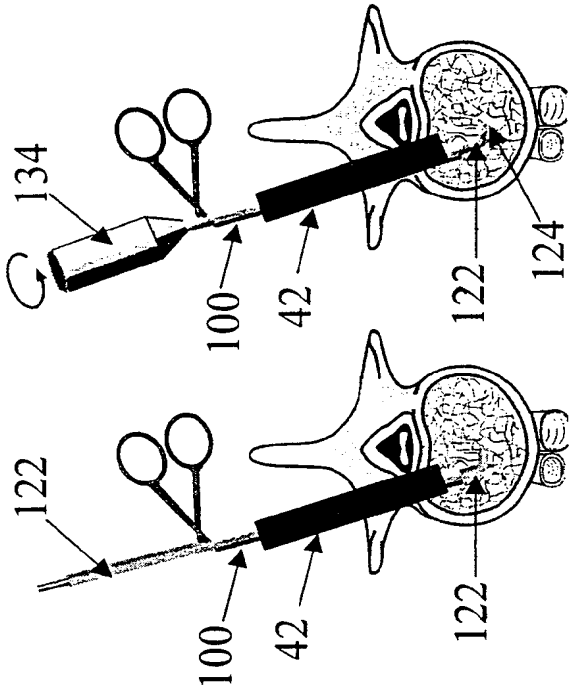

FIG. 10D

SEQUENCE LISTING

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<223> Description of Artificial Sequence: IgSP-NS-hBMP-2
plasmid

<400> 14
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gggtccataa actctgtgac agtggcaatc actttgcctt tctttctaca ggggtgaatt 180
cgcaagccaa acacaaacag cggaaacgcc ttaagtccag ctgtaagaga caccctttgt 240
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ccttttactg ccacggagaa tgccctttc ctctggctga tcatctgaac tccactaatc 360
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tcccgacaga actcagtgct atctcgatgc tgtaccttga cgagaatgaa aaggttgtat 480
taaagaacta tcaggacatg gttgtggagg gttgtgggtg tcgctaggat ccggg 535

<210> 15
<211> 541
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: IgSP-KR-hBMP-2
plasmid

<400> 15
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gggtccataa actctgtgac agtggcaatc actttgcctt tctttctaca ggggtgaatt 180
cgaaacgtca agccaaacac aaacagcgga aacgccttaa gtccagctgt aagagacacc 240
ctttgtacgt ggacttcagt gacgtggggt ggaatgactg gattgtggct cccccggggt 300
atcacgcctt ttactgccac ggagaatgcc cttttcctct ggctgatcat ctgaactcca 360
ctaatcatgc cattgttcag acgttggtca actctgttaa ctctaagatt cctaaggcat 420
gctgtgtccc gacagaactc agtgctatct cgatgctgta ccttgacgag aatgaaaagg 480
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g 541

<210> 16
<211> 547
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
IgSP-RRRR-hBMP-2 plasmid

<400> 16
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gggtccataa actctgtgac agtggcaatc actttgcctt tctttctaca ggggtgaatt 180
cgcgccggcg ccgacaagcc aaacacaaac agcggaaacg ccttaagtcc agctgtaaga 240
gacacccttt gtacgtggac ttcagtgacg tggggtggaa tgactggatt gtggctcccc 300
cggggtatca cgccttttac tgccacggag aatgcccttt tcctctggct gatcatctga 360
actccactaa tcatgccatt gttcagacgt tggtcaactc tgttaactct aagattccta 420

aggcatgctg tgtcccgaca gaactcagtg ctatctcgat gctgtacctt gacgagaatg 480
aaaaggttgt attaaagaac tatcaggaca tggttgtgga gggttgtggg tgtcgctagg 540
atccggg 547